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Der Präsident des Europäischen Patentamts;
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R C van Dijk

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Anmelder/Applicant(s)/Demandeur(s):

Introgene B.V.
Wassenaarseweg 72
2333 AL Leiden
PAYS-BAS

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Gene delivery vectors provided with a tissue tropism for smooth muscle cells and/or endothelial cells

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Title: Gene delivery vectors provided with a tissue tropism for smooth muscle cells, and/or endothelial cells.

FIELD OF THE INVENTION

The invention relates to the field of molecular genetics and medicine. In particular the present invention relates to the field of gene therapy, more in particular to gene therapy using adenoviruses.

BACKGROUND OF THE INVENTION

In gene therapy, genetic information is usually delivered to a host cell in order to either correct (supplement) a genetic deficiency in said cell, or to inhibit an undesired function in said cell, or to eliminate said host cell. Of course the genetic information can also be intended to provide the host cell with a desired function, e.g. to supply a secreted protein to treat other cells of the host, etc.

Many different methods have been developed to introduce new genetic information into cells. Although many different systems may work on cell lines cultured in vitro, only the group of viral vector mediated gene delivery methods seems to be able to meet the required efficiency of gene transfer in vivo. Thus for gene therapy purposes most of the attention is directed toward the development of suitable viral vectors. Today, most of the attention for the development of suitable viral vectors is directed toward those vectors that are based on adenoviruses. These adenovirus vectors can deliver foreign genetic information very efficiently to target cells in vivo. Moreover, obtaining large amounts of adenovirus vectors is for most types of adenovirus vectors not a problem. Adenovirus vectors are relatively easy to concentrate and purify. Moreover, studies in clinical trials have provided valuable information on the use of these vectors in patients.

There are a lot of reasons for using adenovirus vectors for the delivery of nucleic acid to target cells in gene therapy protocols. However, some characteristics of the current vectors limit their use in specific applications. For instance endothelial cells and smooth muscle cells are not easily transduced by the current generation of adenovirus vectors. For many gene therapy applications, such as applications in the cardiovascular area, preferably these types of cells should be genetically modified. On the other hand, in some applications, even the very good in vivo delivery capacity of adenovirus vectors is not sufficient and higher transfer efficiencies are required. This is the case, for instance, when most cells of a target tissue need to be transduced.

The present invention was made in the course of the manipulation of adenovirus vectors. In the following section therefore a brief introduction to adenoviruses is given.

Adenoviruses

Adenoviruses contain a linear double-stranded DNA molecule of approximately 36000 base pairs. It contains identical Inverted Terminal Repeats (ITR) of approximately 90-140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the genome ends. The transcription units are divided in early and late regions. Shortly after infection the E1A and E1B proteins are expressed and function in transactivation of cellular and adenoviral genes. The early regions E2A and E2B encode proteins (DNA binding protein, pre-terminal protein and polymerase) required for the replication of the adenoviral genome (reviewed in van der Vliet, 1995). The early region E4 encodes several proteins with pleiotropic functions e.g. transactivation of the E2 early promoter, facilitating transport and accumulation of viral mRNAs in the late phase of infection and increasing

nuclear stability of major late pre-mRNAs (reviewed in Leppard, 1997). The early region 3 encodes proteins that are involved in modulation of the immune response of the host (Wold *et al*, 1995). The late region is transcribed from one single promoter (major late promoter) and is activated at the onset of DNA replication. Complex splicing and poly-adenylation mechanisms give rise to more than 12 RNA species coding for core proteins, capsid proteins (penton, hexon, fiber and associated proteins), viral protease and proteins necessary for the assembly of the capsid and shut-down of host protein translation(Imperiale, M.J., Akusjnarvi, G. and Leppard, K.N. (1995) Post-transcriptional control of adenovirus gene expression. In: The molecular repertoire of adenoviruses I. P139-171. W. Doerfler and P. Bohm (eds), Springer-Verlag Berlin Heidelberg).

Interaction between virus and host cell

The interaction of the virus with the host cell has mainly been investigated with the serotype C viruses Ad2 and Ad5. Binding occurs via interaction of the knob region of the protruding fiber with a cellular receptor. The receptor for Ad2 and Ad5 and probably more adenoviruses is known as the `Coxsackievirus and Adenovirus Receptor' or CAR protein (Bergelson *et al*, 1997). Internalisation is mediated through interaction of the RGD sequence present in the penton base with cellular integrins (Wickham *et al*, 1993). This may not be true for all serotypes, for example serotype 40 and 41 do not contain a RGD sequence in their penton base sequence (Kidd et al, 1993).

The fiber protein

The initial step for successful infection is binding of adenovirus to its target cell, a process mediated through fiber protein. The fiber protein has a trimeric structure (Stouten et al, 1992) with different lengths depending on

the virus serotype (Signas et al, 1985; Kidd et al, 1993). Different serotypes have polypeptides with structurally similar N and C termini, but different middle stem regions. The first 30 amino acids at the N terminus are involved in anchoring of the fiber to the penton base (Chroboczek et al, 1995), especially the conserved FNPVYP region in the tail (Arnberg et al, 1997). The C-terminus, or knob, is responsible for initial interaction with the cellular adenovirus receptor. After this initial binding secondary binding between the capsid penton base and cell-surface integrins leads to internalisation of viral particles in coated pits and endocytosis (Morgan et al, 1969; Svensson and Persson, 1984; Varga et al, 1991; Greber et al, 1993; Wickham et al, 1993). Integrins are αβ-heterodimers of which at least 14 α-subunits and 8 ß-subunits have been identified (Hynes, 1992). The array of integrins expressed in cells is complex and will vary between cell types and cellular environment. Although the knob contains some conserved regions, between serotypes, knob proteins show a high degree of variability, indicating that different adenovirus receptors exist.

Adenoviral serotypes

At present, six different subgroups of human adenoviruses have been proposed which in total encompass approximately 50 distinct adenovirus serotypes. Besides these human adenoviruses, many animal adenoviruses have been identified (see e.g. Ishibashi and Yasue, 1984). A serotype is defined on the basis of its immunological distinctiveness as determined by quantitative neutralization with animal antiserum (horse, rabbit). If neutralization shows a certain degree of cross-reaction between two viruses, distinctiveness of serotype is assumed if A) the hemagglutinins are unrelated, as shown by lack of cross-reaction on hemagglutination-inhibition, or B) substantial biophysical/biochemical differences in DNA exist (Francki et

al, 1991). The serotypes identified last (42-49) were isolated for the first time from HIV infected patients (Hierholzer et al, 1988; Schnurr et al, 1993). For reasons not well understood, most of such immuno-compromised patients shed adenoviruses that were never isolated from immuno-competent individuals (Hierholzer et al, 1988, 1992; Khoo et al, 1995).

Besides differences towards the sensitivity against neutralising antibodies of different adenovirus serotypes, adenoviruses in subgroup C such as Ad2 and Ad5 bind to different receptors as compared to adenoviruses from subgroup B such as Ad3 and Ad7 (Defer et al, 1990; Gall *et al*, 1996). Likewise, it was demonstrated that receptor specificity could be altered by exchanging the Ad3 knob protein with the Ad 5 knob protein, and vice versa (Krasnykh et al, 1996; Stevenson et al, 1995, 1997). Serotypes 2, 4,5 and 7 all have a natural affiliation towards lung epithelia and other respiratory tissues. In contrast, serotypes 40 and 41 have a natural affiliation towards the gastrointestinal tract. These serotypes differ in at least capsid proteins (penton-base, hexon), proteins responsible for cell binding (fiber protein), and proteins involved in adenovirus replication. It is unknown to what extend the capsid proteins determine the differences in tropism found between the serotypes. It may very well be that post-infection mechanisms determine cell type specificity of adenoviruses. It has been shown that adenoviruses from serotypes A (Ad12 and Ad31),C (Ad2 and Ad5),D (Ad9 and Ad15),E (Ad4) and F (Ad41) all are able to bind labelled, soluble CAR (sCAR) protein when immobilised on nitrocellulose. Furthermore, binding of adenoviruses from these serotypes to Ramos cells, that express high levels of CAR but lack integrins (Roelvink *et al*, 1996), could be efficiently blocked by addition of sCAR to viruses prior to infection (Roelvink *et al*, 1998). However, the fact that (at least some) members of these

subgroups are able to bind CAR does not exclude that these viruses have different infection efficiencies in various cell types. For example subgroup D serotypes have relatively short fiber shafts compared to subgroup A and C viruses. It has been postulated that the tropism of subgroup D viruses is to a large extend determined by the penton base binding to integrins (Roelvink *et al*, 1996; Roelvink *et al*, 1998). Another example is provided by Zabner *et al*, 1998 who have tested 14 different serotypes on infection of human ciliated airway epithelia (CAE) and found that serotype 17 (subgroup D) was bound and internalised more efficiently then all other viruses, including other members of subgroup D. Similar experiments using serotypes from subgroup A-F in primary foetal rat cells showed that adenoviruses from subgroup A and B were inefficient whereas viruses from subgroup D were most efficient (Law *et al*, 1998). Also in this case viruses within one subgroup displayed different efficiencies. The importance of fiber binding for the improved infection of Ad17 in CAE was shown by Armentano *et al* (WO 98/22609) who made a recombinant LacZ Ad2 virus with a fiber gene from Ad17 and showed that the chimaeric virus infected CAE more efficient then LacZ Ad2 viruses with Ad2 fibers.

Thus despite their shared ability to bind CAR, differences in the length of the fiber, knob sequence and other capsid proteins e.g. penton base of the different serotypes may determine the efficiency by which an adenovirus infects a certain target cell. Of interest in this respect is the ability of Ad5 and Ad2 fibers but not of Ad3 fibers to bind to fibronectin III and MHC class 1 α2 derived peptides. This suggests that adenoviruses are able to use cellular receptors other than CAR (Hong *et al*, 1997). Serotypes 40 and 41 (subgroup F) are known to carry two fiber proteins differing in the length of the shaft. The long shafted 41L fiber is shown to bind CAR whereas the

short shafted 41S is not capable of binding CAR (Roelvink *et al*, 1998). The receptor for the short fiber is not known.

Adenoviral gene delivery vectors

Most adenoviral gene delivery vectors currently used in gene therapy are derived from the serotype C adenoviruses Ad2 or Ad5. The vectors have a deletion in the E1 region, where novel genetic information can be introduced. The E1 deletion renders the recombinant virus replication defective. It has been demonstrated extensively that recombinant adenovirus, in particular serotype 5 is suitable for efficient transfer of genes *in vivo* to the liver, the airway epithelium and solid tumours in animal models and human xenografts in immuno-deficient mice (Bout 1996, 1997; Blaese *et al*, 1995).

Gene transfer vectors derived from adenoviruses (adenoviral vectors) have a number of features that make them particularly useful for gene transfer:

1) the biology of the adenoviruses is well characterised,
2) the adenovirus is not associated with severe human pathology,
3) the virus is extremely efficient in introducing its DNA into the host cell,
4) the virus can infect a wide variety of cells and has a broad host-range,
5) the virus can be produced at high titers in large quantities,
6) and the virus can be rendered replication defective by deletion of the early-region 1 (E1) of the viral genome (Brody and Crystal, 1994).

However, there is still a number of drawbacks associated with the use of adenoviral vectors:

1) Adenoviruses, especially the well investigated serotypes Ad2 and Ad5 usually elicit an immune response by the host into which they are introduced,

2) it is currently not feasible to target the virus to certain cells and tissues,

3) the replication and other functions of the adenovirus are not always very well suited for the cells, which are to be provided with the additional genetic material,

4) the serotypes Ad2 or Ad5, are not ideally suited for delivering additional genetic material to organs other than the liver. The liver can be particularly well transduced with vectors derived from Ad2 or Ad5. Delivery of such vectors via the bloodstream leads to a significant deliver of the vectors to the cells of the liver. In therapies were other cell types then liver cells need to be transduced some means of liver exclusion must be applied to prevent uptake of the vector by these cells. Current methods rely on the physical separation of the vector from the liver cells, most of these methods rely on localising the vector and/or the target organ via surgery, balloon angioplasty or direct injection into an organ via for instance needles. Liver exclusion is also being practised through delivery of the vector to compartments in the body that are essentially isolated from the bloodstream thereby preventing transport of the vector to the liver. Although these methods mostly succeed in avoiding gross delivery of the vector to the liver, most of the methods are crude and still have considerable leakage and/or have poor target tissue penetration characteristics. In some cases inadvertent delivery of the vector to liver cells can be toxic to the patient. For instance, delivery of a herpes simplex virus (HSV) thymidine kinase (TK) gene for the subsequent killing of dividing cancer cells through administration of gancyclovir is quite dangerous when also a significant amount of liver cells are transduced by the vector. Significant delivery and subsequent expression of the HSV-TK gene to liver cells is associated with severe toxicity. Thus

there is a discrete need for an inherently safe vector provided with the property of a reduced transduction efficiency of liver cells.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1: Schematic drawing of the pBr/Ad.Bam-rITR construct.
Figure 2: Schematic drawing of the strategy used to delete the fiber gene from the pBr/Ad.Bam-rITR construct.
Figure 3: Schematic drawing of construct pBr/Ad.BamRΔfib.
Figure 4: Sequences of the chimaeric fibers Ad5/12, Ad5/16, Ad5/28, and Ad5/40-L.
Figure 5: Schematic drawing of the construct pClipsal-Luc.
Figure 6: Schematic drawing of the method to generate chimaeric adenoviruses using three overlapping fragments. Early (E) and late regions (L) are indicated. L5 is the fiber coding sequence.
Figure 7: Infection of HUVEC cells using different amount of virus particles/cell and different chimaeric viruses. RLU = relative light units.

Figure 8: Infection of HUVsmc cells using different amounts of virus particles/cell and different chimaeric viruses. RLU = relative light units.
Figure 9: Sequences including the gene encoding adenovirus 16 fiber protein as published in Genbank and sequences including a gene encoding a fiber from an adenovirus 16 variant as isolated in the present invention, wherein the sequences of the fiber protein are from the NdeI-site. Figure 9A nucleotide sequence comparison. Figure 9B amino-acid comparison.

SUMMARY OF THE INVENTION

The present invention provides gene therapy methods, compounds and medicines. The present invention is particularly useful in gene therapy applications were endothelial cells and /or smooth muscle cells form the target cell type. The present invention relates to gene delivery

vehicles provided with a tissue tropism for at least endothelial cells and /or smooth muscle cells. The present invention further relates to gene delivery vehicles having been deprived of a tissue tropism for liver cells.

DETAILED DESCRIPTION OF THE INVENTION.

It is an object of the current invention to provide materials and methods to overcome the limitations of adenoviral vectors mentioned above. In a broad sense, the invention provides new adenoviruses, derived in whole or in part from serotypes different from Ad5. Specific genes of serotypes with preferred characteristics may be combined in a chimaeric vector to give rise to a vector that is better suited for specific applications. Preferred characteristics include, but are not limited to, improved infection of a specific target cell, reduced infection of non-target cells, improved stability of the virus, reduced uptake in antigen presenting cells (APC), or increased uptake in APC, reduced toxicity to target cells, reduced neutralization in humans or animals, reduced or increased CTL response in humans or animals, better and/or prolonged transgene expression, increased penetration capacity in tissues, improved yields in packaging cell lines, etc.

One aspect of the present invention facilitates the combination of the low immunogenicity of some adenoviruses with the characteristics of other adenoviruses that allow efficient gene therapy. Such characteristics may be a high specificity for certain host cells, a good replication machinery for certain cells, a high rate of infection in certain host cells, low infection efficiency in non-target cells, high or low efficiency of APC infection, etc. The invention thus may provide chimaeric adenoviruses having the useful properties of at least two adenoviruses of different serotypes.

Typically, two or more requirements from the above non-exhaustive list are required to obtain an adenovirus capable of efficiently transferring genetic material to a host cell. Therefore the present invention provides adenovirus derived vectors which can be used as cassettes to insert different adenoviral genes from different adenoviral serotypes at the required sites. This way one can obtain a vector capable of producing a chimaeric adenovirus, whereby of course also a gene of interest can be inserted (for instance at the site of E1 of the original adenovirus). In this manner the chimaeric adenovirus to be produced can be adapted to the requirements and needs of certain hosts in need of gene therapy for certain disorders. To enable this virus production, a packaging cell will generally be needed in order to produce sufficient amount of safe chimaeric adenoviruses.

In one of its aspects the present invention provides adenoviral vectors comprising at least a fragment of a fiber protein of an adenovirus from subgroup B. Said fiber protein may be the native fiber protein of the adenoviral vector or may be derived from a serotype different from the serotype the adenoviral vector is based on. In the latter case the adenoviral vector according to the invention is a chimaeric adenovirus displaying at least a fragment of the fiber protein derived from subgroup B adenoviruses that fragment comprising at least the receptor binding sequence. Typically such a virus will be produced using a vector (typically a plasmid, a cosmid or baculovirus vector). Such vectors are also subject of the present invention. A preferred vector is a vector that can be used to make a chimaeric recombinant virus specifically adapted to the host to be treated and the disorder to be treated.

The present invention also provides a chimaeric adenovirus based on adenovirus type 5 but having at least a fragment of the fiber sequence from adenovirus type 16, whereby the fragment of the fiber of Ad16 comprises the fragment of the fiber protein that is involved in binding a host cell.

The present invention also provides chimaeric adenoviral vectors that show improved infection as compared to adenoviruses from other subgroups in specific host cells for example, but not limited to, endothelial cells and smooth muscle cells of human or animal origin. An important feature of the present invention is the means to produce the chimaeric virus. Typically, one does not want an adenovirus batch to be administered to the host cell, which contains replication competent adenovirus. In general therefore it is desired to omit a number of genes (but at least one) from the adenoviral genome on the vector encoding the chimaeric virus and to supply these genes in the genome of the cell in which the vector is brought to produce chimaeric adenovirus. Such a cell is usually called a packaging cell. The invention thus also provides a packaging cell for producing a chimaeric adenovirus according to the invention, comprising in trans all elements necessary for adenovirus production not present on the adenoviral vector according to the invention. Typically vector and packaging cell have to be adapted to one another in that they have all the necessary elements, but that they do not have overlapping elements which lead to replication competent virus by recombination. Thus the invention also provides a kit of parts comprising a packaging cell according to the invention and a recombinant vector according the invention whereby there is essentially no sequence overlap leading to recombination resulting in the production of replication competent adenovirus between said cell and said vector. For certain applications for example when the therapy is aimed at eradication of tumour cells, the adenoviral vector according to the invention may be replication competent or capable of replicating under certain conditions for example in specific cell types like tumour cells or tumour endothelial cells.

It is within the scope of the invention to insert more genes, or a functional part of these genes from the same or other serotypes into the adenoviral vector replacing the

corresponding native sequences. Thus for example replacement of (a functional part of the) fiber sequences with corresponding sequences of other serotypes may be combined with for example replacements of (a functional part of) other capsid genes like penton base or hexon with corresponding sequences of said serotype or of other distinct serotypes. Persons skilled in the art understand that other combinations not limited to the said genes are possible and are within the scope of the invention. The chimaeric adenoviral vector according to the invention may originate from at least two different serotypes. This may provide the vector with preferred characteristics such as improved infection of target cells and/or less infection of non-target cells, improved stability of the virus, reduced immunogenicity in humans or animals (e.g. reduced uptake in APC, reduced neutralisation in the host and/or reduced cytotoxic T-lymphocyte (CTL) response), increased penetration of tissue, better longevity of transgene expression, etc. In this aspect it is preferred to use capsid genes e.g. penton and/or hexon genes from less immunogenic serotypes as defined by the absence or the presence of low amounts of neutralising antibodies in the vast majority of hosts. It is also preferred to use fiber and/or penton sequences from serotypes that show improved binding and internalisation in the target cells. Furthermore it is preferred to delete from the viral vector those genes which lead to expression of adenoviral genes in the target cells. In this aspect a vector deleted of all adenoviral genes is also preferred. Furthermore it is preferred that the promoter driving the gene of interest to be expressed in the target cells is a cell type specific promoter.

In order to be able to precisely adapt the viral vector and provide the chimaeric virus with the desired properties at will, it is preferred that a library of adenoviral genes is provided whereby the genes to be exchanged are located on plasmid- or cosmid-based adenoviral constructs whereby the

genes or the sequences to be exchanged are flanked by restriction sites. The preferred genes or sequences can be selected from the library and inserted in the adenoviral constructs that are used to generate the viruses. Typically such a method comprises a number of restriction and ligation steps and transfection of a packaging cell. The adenoviral vector can be transfected in one piece, or as two or more overlapping fragments, whereby viruses are generated by homologous recombination. For example the adenoviral vector may be built up from two or more overlapping sequences for insertion or replacements of a gene of interest in for example the E1 region, for insertion or replacements in penton and/or hexon sequences, and for insertions or replacements into fiber sequences. Thus the invention provides a method for producing chimaeric adenoviruses having one or more desired properties like a desired host range and diminished antigenicity, comprising providing one or more vectors according to the invention having the desired insertion sites, inserting into said vectors at least a functional part of a fiber protein derived from an adenovirus serotype having the desired host range and/or inserting a functional part of a capsid protein derived from an adenovirus serotype having relatively low antigenicity and transfecting said vectors in a packaging cell according to the invention and allowing for production of chimaeric viral particles. Of course other combinations of other viral genes originating from different serotypes can also be inserted as disclosed herein before. Chimaeric viruses having only one non-native sequence in addition to an insertion or replacement of a gene of interest in the E1 region, are also within the scope of the invention.
An immunogenic response to adenovirus that typically occurs is the production of neutralising antibodies by the host. This is typically a reason for selecting a capsid protein like penton, hexon and/or fiber of a less immunogenic serotype.

Of course it may not be necessary to make chimaeric adenoviruses which have complete proteins from different serotypes. It is well within the skill of the art to produce chimaeric proteins, for instance in the case of fiber proteins it is very well possible to have the base of one serotype and the shaft and the knob from another serotype. In this manner it becomes possible to have the parts of the protein responsible for assembly of viral particles originate from one serotype, thereby enhancing the production of intact viral particles. Thus the invention also provides a chimaeric adenovirus according to the invention, wherein the hexon, penton, fiber and/or other capsid proteins are chimaeric proteins originating from different adenovirus serotypes. Besides generating chimaeric adenoviruses by swapping entire wild type capsid (protein) genes etc. or parts thereof, it is also within the scope of the present invention to insert capsid (protein) genes etc. carrying non-adenoviral sequences or mutations such as point mutations, deletions, insertions, etc. which can be easily screened for preferred characteristics such as temperature stability, assembly, anchoring, redirected infection, altered immune response etc. Again other chimaeric combinations can also be produced and are within the scope of the present invention.

It has been demonstrated in mice and rats that upon in vivo systemic delivery of recombinant adenovirus of common used serotypes for gene therapy purposes more than 90% of the virus is trapped in the liver (Herz et al, 1993; Kass-Eisler et al, 1994; Huard et al, 1995). It is also known that human hepatocytes are efficiently transduced by adenovirus serotype 5 vectors (Castell, J.V., Hernandez, D. Gomez-Foix, A.M., Guillen, I, Donato, T. and Gomez-Lechon, M.J. (1997). Adenovirus-mediated gene transfer into human hepatocytes: analysis of the biochemical functionality of transduced

cells. Gene Ther. 4(5), p455-464). Thus in vivo gene therapy by systemic delivery of Ad2 or Ad5 based vectors is seriously hampered by the efficient uptake of the viruses in the liver leading to unwanted toxicity and less virus being available for transduction of the target cells. Therefore, alteration of the adenovirus serotype 5 host cell range to be able to target other organs in vivo is a major interest of the invention.

To obtain re-directed infection of recombinant adenovirus serotype 5, several approaches have been or still are under investigation. Wickham et al have altered the RGD (Arg, Gly, Asp) motif in the penton base which is believed to be responsible for the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin binding to the penton base. They have replaced this RGD motif by another peptide motif which is specific for the $\alpha_4\beta_1$ receptor. In this way targeting the adenovirus to a specific target cell could be accomplished (Wickham et al, 1995). Krasnykh et al (1998) have made use of the HI loop available in the knob. This loop is, based on X-ray crystallography, located on the outside of the knob trimeric structure and therefore is thought not to contribute to the intramolecular interactions in the knob. Insertion of a FLAG coding sequence into the HI loop resulted in fiber proteins that were able to trimerise and it was further shown that viruses containing the FLAG sequence in the knob region could be made. Although interactions of the FLAG-containing knob with CAR are not changed, insertion of ligands in the HI loop may lead to retargeting of infection. Although successful introduction of changes in the adenovirus serotype 5 fiber and penton-base have been reported, the complex structure of knob and the limited knowledge of the precise amino acids interacting with CAR render such targeting approaches laborious and difficult. The use of antibodies binding to CAR and to a specific cellular receptor has also been described (Wickham et al, 1996; Rogers et al, 1997). This approach is however

limited by the availability of a specific antibody and by the complexity of the gene therapy product.
To overcome the limitations described above we used pre-existing adenovirus fibers, penton base proteins, hexon proteins or other capsid proteins derived from other adenovirus serotypes. By generating chimaeric adenovirus serotype 5 libraries containing structural proteins of alternative adenovirus serotypes, we have developed a technology, which enables rapid screening for a recombinant adenoviral vector with preferred characteristics.

It is an object of the present invention to provide methods for the generation of chimaeric capsids which can be targeted to specific cell types *in vitro* as well as *in vivo*, and thus have an altered tropism for certain cell types. It is a further object of the present invention to provide methods and means by which an adenovirus or an adenovirus capsid can be used as a protein or nucleic acid delivery vehicle to a specific cell type or tissue.
The generation of chimaeric adenoviruses based on adenovirus serotype 5 with modified late genes is described. For this purpose, three plasmids, which together contain the complete adenovirus serotype 5 genome, were constructed. From one of these plasmids part of the DNA encoding the adenovirus serotype 5 fiber protein was removed and replaced by linker DNA sequences that facilitate easy cloning. This plasmid subsequently served as template for the insertion of DNA encoding fiber protein derived from different adenovirus serotypes. The DNA sequences derived from the different serotypes were obtained using the polymerase chain reaction technique in combination with (degenerate) oligonucleotides. At the former E1 location in the genome of adenovirus serotype 5, any gene of interest can be cloned. A single transfection procedure of the three plasmids together results in the formation of a recombinant chimaeric adenovirus. Alternatively, cloning of the sequences obtained

from the library of genes can be such that the chimaeric adenoviral vector is build up from one or two fragments. For example one construct contains at least the left ITR and sequences necessary for packaging of the virus, an expression cassette for the gene of interest and sequences overlapping with the second construct comprising all sequences necessary for replication and virus formation not present in the packaging cell as well as the non-native sequences providing the preferred characteristics. This new technology of libraries consisting of chimaeric adenoviruses thus allows for a rapid screening for improved recombinant adenoviral vectors for *in vitro* and *in vivo* gene therapy purposes.

The use of adenovirus type 5 for in vivo gene therapy is limited by the apparent inability to infect certain cell types e.g. human endothelial cells and smooth muscle cells and the preference of infection of certain organs e.g. liver and spleen. Specifically this has consequences for treatment of cardiovascular diseases like restenosis and pulmonary hypertension. Adenovirus-mediated delivery of human ceNOS (constitutive endothelial nitric oxide synthase) has been proposed as treatment for restenosis after percutaneous transluminal coronary angioplasty (PTCA). Restenosis is characterised by progressive arterial remodeling, extracellular matrix formation and intimal hyperplasia at the site of angioplasty (Schwartz *et al*, 1993; Carter *et al*, 1994; Shi *et al*,1996). NO is one of the vasoactive factors shown to be lost after PTCA-induced injury to the endothelial barrier (Lloyd Jones and Bloch, 1996). Thus restoration of NO levels after balloon-induced injury by means of adenoviral delivery of ceNOS may prevent restenosis (Varenne *et al*, 1998). Other applications for gene therapy whereby the viruses or chimaeric viruses according to the invention are superior to Ad2 or Ad5 based viruses, given as non-limiting examples, are production of proteins by

endothelial cells that are secreted into the blood, treatment of hypertension, preventive treatment of stenosis during vein grafting, angiogenesis, heart failure, renal hypertension and others.

In one embodiment this invention describes adenoviral vectors that are, amongst others, especially suited for gene delivery to endothelial cells and smooth muscle cells important for treatment of cardiovascular disorders. The adenoviral vectors preferably are derived from subgroup B adenoviruses or contain at least a functional part of the fiber protein from an adenovirus from subgroup B comprising at least the cell-binding moiety of the fiber protein.
In a further preferred embodiment the adenoviral vectors are chimaeric vectors based on adenovirus type 5 and contain at least a functional part of the fiber protein from adenovirus type 16.
In another embodiment this invention provides adenoviral vectors or chimaeric adenoviral vectors that escape the liver following systemic administration. Preferably these adenoviral vectors are derived from subgroup A, B, D, or F in particular serotypes 12, 16, 28 and 40 or contain at least the cell-binding moiety of the fiber protein derived from said adenoviruses.
It is to be understood that in all embodiments the adenoviral vectors may be derived from the serotype having the desired properties or that the adenoviral vector is based on an adenovirus from one serotype and contains the sequences comprising the desired functions of another serotype, these sequences replacing the native sequences in the said serotype.

In another aspect this invention describes chimaeric adenoviruses and methods to generate these viruses that have an altered tropism different from that of adenovirus serotype 5. For example, viruses based on adenovirus serotype 5 but displaying any adenovirus

fiber existing in nature. This chimaeric adenovirus serotype 5 is able to infect certain cell types more efficiently, or less efficiently *in vitro* and *in vivo* than the adenovirus serotype 5. Such cells include but are not limited to endothelial cells, smooth muscle cells, dendritic cells, neuronal cells, glial cells, synovical cells, lung epithelial cells, hemopoietic stem cells, monocytic/macrophage cells, tumour cells, skeletal muscle cells, mesothelial cells, synoviocytes, etc.

In another aspect the invention describes the construction and use of libraries consisting of distinct parts of adenovirus serotype 5 in which one or more genes or sequences have been replaced with DNA derived from alternative human or animal serotypes. This set of constructs, in total encompassing the complete adenovirus genome, allows for the construction of unique chimaeric adenoviruses customised for a certain disease, group of patients or even a single individual.
In all aspects of the invention the chimaeric adenoviruses may, or may not, contain deletions in the E1 region and insertions of heterologous genes linked either or not to a promoter. Furthermore, chimaeric adenoviruses may, or may not, contain deletions in the E3 region and insertions of heterologous genes linked to a promoter. Furthermore, chimaeric adenoviruses may, or may not, contain deletions in the E2 and/or E4 region and insertions of heterologous genes linked to a promoter. In the latter case E2 and/or E4 complementing cell lines are required to generate recombinant adenoviruses. In fact any gene in the genome of the viral vector can be taken out and supplied in trans. Thus, in the extreme situation, chimaeric viruses do not contain any adenoviral genes in their genome and are by definition minimal adenoviral vectors. In this case all adenoviral functions are supplied in trans using stable cell

lines and/or transient expression of these genes. A method for producing minimal adenoviral vectors is described in WO97/00326 and is taken as reference herein. In another case Ad/AAV chimaeric molecules are packaged into the adenovirus capsids of the invention. A method for producing Ad/AAV chimaeric vectors is described in EP 97204085.1 and is taken as reference herein. In principle any nucleic acid may be provided with the adenovirus capsids of the invention.

In one embodiment the invention provides a gene delivery vehicle having been provided with at least a tissue tropism for smooth muscle cells and/or endothelial cells. In another embodiment the invention provides a gene delivery vehicle deprived of a tissue tropism for at least liver cells. Preferably, said gene delivery vehicle is provided with a tissue tropism for at least smooth muscle cells and/or endothelial cells and deprived of a tissue tropism for at least liver cells. In a preferred embodiment of the invention said gene delivery vehicle is provided with a tissue tropism for at least smooth muscle cells and/or endothelial cells and/or deprived of a tissue tropism for at least liver cells using a fiber protein derived from a subgroup B adenovirus, preferably of adenovirus 16. In a preferred aspect of the invention said gene delivery vehicle comprises a virus capsid. Preferably said virus capsid comprises a virus capsid derived in whole or in part from an adenovirus of subgroup B, preferably from adenovirus 16, or it comprises proteins, or parts thereof, from an adenovirus of subgroup B, preferably of adenovirus 16. In preferred embodiment of the invention said virus capsid comprises proteins, or fragments thereof, from at least two different viruses, preferably adenoviruses. In a preferred embodiment of this aspect of the invention at least one of said virus is an adenovirus of subgroup B, preferably adenovirus 16.

In a preferred embodiment of the invention said gene delivery vehicle comprises an adenovirus fiber protein or fragments thereof. Said fiber protein is preferably derived from an

adenovirus of subgroup B, preferably of adenovirus 16. Said gene delivery vehicle may further comprise other fiber proteins, or fragments thereof, from other adenoviruses. Said gene delivery vehicle may or may not comprise other adenovirus proteins. Nucleic acid may be linked directly to fiber proteins, or fragments thereof, but may also be linked indirectly. Examples of indirect linkages include but are not limited to, packaging of nucleic acid into adenovirus capsids or packaging of nucleic acid into liposomes, wherein a fiber protein, or a fragment thereof, is incorporated into an adenovirus capsid or linked to a liposome. Direct linkage of nucleic acid to a fiber protein, or a fragment thereof, may be performed when said fiber protein, or a fragment thereof, is not part of a complex or when said fiber protein, or a fragment thereof, is part of complex such as an adenovirus capsid.

In one embodiment of the invention is provided a gene delivery vehicle comprising an adenovirus fiber protein wherein said fiber protein comprises a tissue determining fragment of an adenovirus of subgroup B adenovirus preferably of adenovirus 16. Adenovirus fiber protein comprises three functional domains. One domain, the base, is responsible for anchoring the fiber to a penton base of the adenovirus capsid. Another domain, the knob, is responsible for receptor recognition whereas the shaft domain functions as a spacer separating the base from the knob. The different domains may also have other function. For instance, the shaft is presumably also involved in target cell specificity. Each of the domains mentioned above may be used to define a fragment of a fiber. However, fragments may also be identified in another way. For instance the knob domain comprises of a receptor binding fragment and a shaft binding fragment. The base domain comprises of a penton base binding fragment and a shaft binding fragment. Moreover, the shaft comprises of repeated stretches of amino acids. Each of these repeated stretches may be a fragment.

A tissue tropism determining fragment of a fiber protein may be a single fragment of a fiber protein or a combination of fragments of at least one fiber protein, wherein said tissue tropism determining fragment, either alone or in combination with a virus capsid, determines the efficiency with which a gene delivery vehicle can transduce a given cell or cell type, preferably but not necessarily in a positive way. With a tissue tropism for liver cells is meant a tissue tropism for cells residing in the liver, preferably liver parenchyma cells.

A tissue tropism for a certain tissue may be provided by increasing the efficiency with which cells of said tissue are transduced, alternatively, a tissue tropism for a certain tissue may be provided by decreasing the efficiency with which other cells than the cells of said tissue are transduced.

Fiber proteins possess tissue tropism determining properties. The most well described fragment of the fiber protein involved in tissue tropism is the knob domain. However, the shaft domain of the fiber protein also possesses tissue tropism determining properties. However, not all of the tissue tropism determining properties of an adenovirus capsid are incorporated into a fiber protein.

In a preferred embodiment of the invention, a fiber protein derived from a subgroup B adenovirus, preferably adenovirus 16, is combined with the non-fiber capsid proteins from an adenovirus of subgroup C, preferably of adenovirus 5.

In one aspect of the invention is provided a gene delivery vehicle comprising a nucleic acid derived from an adenovirus. In a preferred embodiment of the invention said adenovirus nucleic acid comprises at least one nucleic acid sequence encoding a fiber protein comprising at least a tissue tropism determining fragment of a subgroup B adenovirus fiber protein, preferably of adenovirus 16. In a preferred aspect said adenovirus comprises nucleic acid from at least two

different adenoviruses. In a preferred aspect said adenovirus comprises nucleic acid from at least two different adenoviruses wherein at least one nucleic acid sequence encoding a fiber protein comprising at least a tissue tropism determining fragment of a subgroup B adenovirus fiber protein, preferably of adenovirus 16.

In a preferred embodiment of the invention said adenovirus nucleic acid is modified such that the capacity of said adenovirus nucleic acid to replicate in a target cell has been reduced or disabled. This may be achieved through inactivating or deleting genes encoding early region 1 proteins.

In another preferred embodiment said adenovirus nucleic acid is modified such that the capacity of a host immune system to mount an immune response against adenovirus proteins encoded by said adenovirus nucleic acid has been reduced or disabled. This may be achieved through deletion of genes encoding proteins of early region 2 and/or early region 4. Alternatively, genes encoding early region 3 proteins, may be deleted, or on the contrary, considering the anti-immune system function of some of the proteins encoded by the genes in early region 3, the expression of early region 3 proteins may be enhanced for some purposes. Also, the adenovirus nucleic acid may be altered by a combination of two or more of the specific alterations of the adenovirus nucleic acid mentioned above. It is clear that when essential genes are deleted from the adenovirus nucleic acid, the genes must be complemented in the cell that is going to produce the adenovirus nucleic acid, the adenovirus vector, the vehicle or the chimaeric capsid. The adenovirus nucleic acid may also be modified such that the capacity of a host immune system to mount an immune response against adenovirus proteins encoded by said adenovirus nucleic acid has been reduced or disabled, in other ways then mentioned above, for instance through exchanging capsid proteins, or fragments thereof, by capsid proteins, or fragments thereof, from other serotypes for which humans do not have, or have low levels of, neutralising

antibodies. Another example of this is the exchange of genes encoding capsid proteins with the genes encoding for capsid proteins from other serotypes. Also capsid proteins, or fragments thereof, may be exchanged for other capsid proteins, or fragments thereof, for which individuals are not capable of, or have a low capacity of, raising an immune response against.

An adenovirus nucleic acid may be altered further or instead of one or more of the alterations mentioned above, by inactivating or deleting genes encoding adenovirus late proteins such as but not-limited to, hexon, penton, fiber and/or protein IX.

In a preferred embodiment of the invention all genes encoding adenovirus proteins are deleted from said adenovirus nucleic acid, turning said nucleic acid into a minimal adenovirus vector.

In another preferred embodiment of the invention said adenovirus nucleic acid is an Ad/AAV chimaeric vector, wherein at least the integration means of an adeno-associated virus (AAV) are incorporated into said adenovirus nucleic acid.

In a preferred embodiment of the invention, a vector or a nucleic acid, which may be one and the same or not, according to the invention further comprises at least one non-adenovirus gene. Preferably, at least one of said non-adenovirus gene is selected from the group of genes encoding: an apolipoprotein, a ceNOS, a herpes simplex virus thymidine kinase, an interleukin-3, an interleukin-1α, an (anti)angiogenesis protein such as angiostatin, an anti-proliferation protein, a vascular endothelial growth factor (VGAF), a basic fibroblast growth factor (bFGF),a hypoxia inducible factor 1α (HIF-1α), a PAI-1 or a smooth muscle cell anti-migration protein.

In another aspect, the invention provides a cell for the production of a gene delivery vehicle provided with at least a tissue tropism for smooth muscle cells and/or endothelial

cells. In another aspect, the invention provides a cell for the production of a gene delivery vehicle deprived of at least a tissue tropism for liver cells. In another aspect, the invention provides a cell for the production of a gene delivery vehicle provided with at least a tissue tropism for smooth muscle cells and/or endothelial cells and deprived of at least a tissue tropism for liver cells. In a preferred embodiment of the invention said cell is an adenovirus packaging cell, wherein an adenovirus nucleic acid is packaged into an adenovirus capsid. In one aspect of an adenovirus packaging cell of the invention all proteins required for the replication and packaging of an adenovirus nucleic acid, except for the proteins encoded by early region 1, are provided by genes incorporated in said adenovirus nucleic acid. The early region 1 encoded proteins in this aspect of the invention may be encoded by genes incorporated into the cells genomic DNA. In a preferred embodiment of the invention said cell is PER.C6 (ECACC deposit number 96022940). In general, when gene products required for the replication and packaging of adenovirus nucleic acid into adenovirus capsid are not provided by a adenovirus nucleic acid, they are provided by the packaging cell, either by transient transfection, or through stable transformation of said packaging cell. However, a gene product provided by the packaging cell may also be provided by a gene present on said adenovirus nucleic acid. For instance fiber protein may be provided by the packaging cell, for instance through transient transfection, and may be encoded by the adenovirus nucleic acid. This feature can among others be used to generate adenovirus capsids comprising of fiber proteins from two different viruses.

The gene delivery vehicles of the invention are useful for the treatment cardiovascular disease or disease treatable by nucleic acid delivery to endothelial cells or smooth muscle cells. A non-limiting example of the latter is for instance cancer, where the nucleic acid transferred comprises a gene encoding an anti-angiogenesis protein.

The gene delivery vehicles of the invention may be used as a pharmaceutical for the treatment of said diseases. Alternatively, gene delivery vehicles of the invention may be used for the preparation of a medicament for the treatment of said diseases.

In one aspect the invention provides an adenovirus capsid with or provided with a tissue tropism for smooth muscle cells and/or endothelial cells wherein said capsid preferably comprises proteins from at least two different adenoviruses and wherein at least a tissue tropism determining fragment of a fiber protein is derived from a subgroup B adenovirus, preferably of adenovirus 16. In another aspect the invention provides an adenovirus capsid deprived of a tissue tropism for liver cells wherein said capsid preferably comprises proteins from at least two different adenoviruses and wherein at least a tissue tropism determining fragment of a fiber protein is derived from a subgroup B adenovirus, preferably of adenovirus 16.

In one embodiment the invention comprises the use of an adenovirus capsid, for the delivery of nucleic acid to smooth muscle cells and/or endothelial cells. In another embodiment the invention comprises the use of an adenovirus capsid, for preventing the delivery of nucleic acid to liver cells.

The adenovirus capsids of the invention may be used for the treatment cardiovascular disease or disease treatable by nucleic acid delivery to endothelial cells or smooth muscle cells. Example of the latter is for instance cancer where the nucleic acid transferred comprises a gene encoding an anti-angiogenesis protein.

The adenovirus capsids of the invention may be used as a pharmaceutical for the treatment of said diseases. Alternatively, adenovirus capsids of the invention may be used for the preparation of a medicament for the treatment of said diseases.

In another aspect of the invention is provided construct pBr/Ad.BamRΔFib, comprising adenovirus 5 sequences 21562-31094 and 32794-35938.

In another aspect of the invention is provided construct pBr/AdBamRfib16, comprising adenovirus 5 sequences 21562-31094 and 32794-35938, further comprising an adenovirus 16 gene encoding fiber protein.

In yet another aspect of the invention is provided construct pBr/AdBamR.pac/fib16, comprising adenovirus 5 sequences 21562-31094 and 32794-35938, further comprising an adenovirus 16 gene encoding fiber protein, and further comprising a unique PacI-site in the proximity of the adenovirus 5 right terminal repeat, in the non-adenovirus sequence backbone of said construct.

In another aspect of the invention is provided construct pWE/Ad.AflIIrITRfib16 comprising Ad5 sequence 3534-31094 and 32794-35938, further comprising an adenovirus 16 gene encoding fiber protein.

In another aspect of the invention is provided construct pWE/Ad.AflIIrITRDE2Afib16 comprising Ad5 sequences 3534-22443 and 24033-31094 and 32794-35938, further comprising an adenovirus 16 gene encoding fiber protein.

In the numbering of the sequences mentioned above, the number is depicted until and not until plus.

In a preferred embodiment of the invention said constructs are used for the generation of a gene delivery vehicle or an adenovirus capsid with a tissue tropism for smooth muscle cells and/or endothelial cells.

In another aspect the invention provides a library of adenovirus vectors, or gene delivery vehicles which may be one and the same or not, comprising a large selection of non-adenovirus nucleic acids. In another aspect of the invention, adenovirus genes encoding capsid proteins are used to generate a library of adenovirus capsids comprising of proteins derived from at least two different adenoviruses, said adenoviruses preferably being derived from two different serotypes, wherein preferably one serotype is an adenovirus of subgroup B. In a particularly preferred embodiment of the invention a library of adenovirus capsids is generated

comprising proteins from at least two different adenoviruses and wherein at least a tissue tropism determining fragment of fiber protein is derived from an adenovirus of subgroup B, preferably of adenovirus 16.

A fiber protein of adenovirus 16 preferably comprises of the sequence given in figure 9. However within the scope of the present invention analogous sequences may be obtained through using codon degeneracy. Alternatively, amino-acid substitutions or insertions or deletions may be performed as long as the tissue tropism determining property is not significantly altered. Such amino-acid substitutions may be within the same polarity group or without.

In the following the invention is illustrated by a number of non-limiting examples.

EXAMPLES

Example 1: Generation of adenovirus serotype 5 based viruses with chimaeric fiber proteins

Generation of adenovirus template clones lacking DNA encoding for fiber

The fiber coding sequence of adenovirus serotype 5 is located between nucleotides 31042 and 32787. To remove the adenovirus serotype 5 DNA encoding fiber we started with construct pBr/Ad.Bam-rITR (Figure 1; ECACC deposit P97082122). From this construct first a NdeI site was removed. For this purpose, pBr322 plasmid DNA was digested with NdeI after which protruding ends were filled using Klenow enzyme. This pBr322 plasmid was then re-ligated, digested with NdeI and transformed into *E. coli* DH5α. The obtained pBr/ΔNdeI plasmid was digested with ScaI and SalI and the resulting 3198 bp vector fragment was ligated to the 15349 bp ScaI-SalI fragment derived from pBr/Ad.BamrITR, resulting in plasmid pBr/Ad.Bam-rITRΔNdeI which hence contained a unique NdeI site. Next a PCR was performed with oligonucleotides "NY-up" and "NY-down" (Figure 2). During amplification, both a NdeI and a NsiI restriction site were introduced to facilitate cloning of the amplified fiber DNAs. Amplification consisted of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45 sec. at 72°C. The PCR reaction contained 25 pmol of oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with 1.5 mM $MgCl_2$, and 1 unit of Elongase heat stable polymerase (Gibco, The Netherlands). One-tenth of the PCR product was run on an agarose gel which demonstrated that the expected DNA fragment of ± 2200 bp was amplified. This PCR fragment was subsequently purified using Geneclean kit system.(Bio101 Inc.) Then, both the construct pBr/Ad.Bam-rITRΔNdeI as well as the PCR product were digested with restriction enzymes NdeI and SbfI. The PCR fragment was subsequently cloned using

T4 ligase enzyme into the NdeI and SbfI sites thus generating pBr/Ad.BamRΔFib (Figure 3).

Amplification of fiber sequences from adenovirus serotypes

To enable amplification of the DNAs encoding fiber protein derived from alternative serotypes degenerate oligonucleotides were synthesised. For this purpose, first known DNA sequences encoding for fiber protein of alternative serotypes were aligned to identify conserved regions in both the tail region as well as the knob region of the fiber protein. From the alignment, which contained the nucleotide sequence of 19 different serotypes representing all 6 subgroups, (degenerate) oligonucleotides were synthesised (see Table I). Also shown in table 3 is the combination of oligonucleotides used to amplify the DNA encoding fiber protein of a specific serotype. The amplification reaction (50 µl) contained 2 mM dNTPs, 25 pmol of each oligonucleotide, standard 1x PCR buffer, 1,5 mM $MgCl_2$, and 1 Unit Pwo heat stable polymerase (Boehringer Mannheim) per reaction. The cycler program contained 20 cycles, each consisting of 30 sec. 94°C, 60 sec. 60-64°C, and 120 sec. 72°C. One-tenth of the PCR product was run on an agarose gel to demonstrate that a DNA fragment was amplified. Of each different template, two independent PCR reactions were performed.

Generation of chimaeric adenoviral DNA constructs

All amplified fiber DNAs as well as the vector (pBr/Ad.BamRΔFib) were digested with NdeI and NsiI. The digested DNAs were subsequently run on a agarose gel after which the fragments were isolated from the gel and purified using the Geneclean kit (Bio101 Inc). The PCR fragments were then cloned into the NdeI and NsiI sites of pBr/AdBamRΔFib, thus generating pBr/AdBamRFibXX (where XX stands for the

serotype number of which the fiber DNA was isolated). The inserts generated by PCR were sequenced to confirm correct amplification. The obtained sequences of the different fiber genes are shown in Figure 4.

Generation of recombinant adenovirus chimaeric for fiber protein

To enable efficient generation of chimaeric viruses an AvrII fragment from the pBr/AdBamRFib16, pBr/AdBamRFib28, pBr/AdBamRFib40-L constructs was subcloned into the vector pBr/Ad.Bam-rITR.pac#8 (ECACC deposit #P97082121) replacing the corresponding sequences in this vector. pBr/Ad.Bam-rITR.pac#8 has the same adenoviral insert as pBr/Ad.Bam-rITR but has a PacI site near the rITR that enables the ITR to be separated from the vector sequences. The construct pWE/Ad.AflII-Eco was generated as follows. PWE.pac was digested with ClaI and the 5 prime protruding ends were filled in with klenow enzyme. The DNA was then digested with PacI and isolate from agarose gel. PWE/AflIIrITR was digested with EcoRI and after treatment with klenow enzyme digested with PacI. The large 24 kb. fragment containing the adenoviral sequences was isolated from agarose gel and ligated to the ClaI digested and blunted pWE.Pac vector. Use was made of the ligation express kit from Clontech. After transformation of XL10-gold cells from Stratagene, clones were identified that contained the expected construct. PWE/Ad.AlfII-Eco contains Ad5 sequences from basepairs 3534-27336. Three constructs, pClipsal-Luc (Figure 5) digested with SalI, pWE/Ad.AflII-Eco digested with PacI and EcoRI and pBr/AdBamR.pac/fibXX digested with BamHI and PacI were transfected into adenovirus producer cells (PER.C6, Fallaux *et al*, 1998). Figure 6 schematically depicts the method and fragments used to generate the chimaeric viruses. Only pBr/Ad.BamRfib12 was used without subcloning in the PacI containing vector and therefor was not liberated from vector sequences using PacI but was digested with ClaI which leaves

approximately 160 bp of vector sequences attached to the right ITR. Furthermore, the pBr/Ad.BamRfib12 and pBr/Ad.BamRfib28 contain an internal BamHI site in the fiber sequences and were therefor digested with SalI which cuts in the vector sequences flanking the BamHI site. For transfection, 2 µg of pCLIPsal-Luc, and 4 µg of both pWE/Ad.AflII-Eco and pBr/AdBamR.pac/fibXX were diluted in serum free DMEM to 100 µl total volume. To this DNA suspension 100 µl 2.5x diluted lipofectamine (Gibco) in serum-free medium was added. After 30 minutes at room temperature the DNA-lipofectamine complex solution was added to 2.5 ml of serum-free DMEM which was subsequently added to a T25 cm^2 tissue culture flask. This flask contained PER.C6 cells that were seeded 24-hours prior to transfection at a density of $1x10^6$ cells/flask. Two hours later, the DNA-lipofectamine complex containing medium was diluted once by the addition of 2.5 ml DMEM supplemented with 20% foetal calf serum. Again 24 hours later the medium was replaced by fresh DMEM supplemented with 10% foetal calf serum. Cells were cultured for 6-8 days, subsequently harvested, and freeze/thawed 3 times. Cellular debris was removed by centrifugation for 5 minutes at 3000 rpm room temperature. Of the supernatant (12.5 ml) 3-5 ml was used to infect again PER.C6 cells (T80 cm^2 tissue culture flasks). This re-infection results in full cytopathogenic effect (CPE) after 5-6 days after which the adenovirus is harvested as described above.

Production of chimaeric adenoviruses

10 ml of the above crude cell lysate was used to inoculate a 1 litre fermentor which contained 1 - 1.5 x 10^6 PER.C6 cells/ml growing in suspension. Three days after inoculation, the cells were harvested and pelleted by centrifugating for 10 min at 1750 rpm at room temperature (RT). Adenovirus present in the pelleted cells was subsequently extracted and

purified using the following downstream processing protocol. The pellet was dissolved in 50 ml 10 mM $NaPO_4^-$ and frozen at -20°C. After thawing at 37°C, 5.6 ml deoxycholate (5% w/v) was added. The solution was mixed and incubated for 15 minutes at 37°C to completely lyse the cells. After homogenizing the solution, 1875 µl 1M $MgCl_2$ and 5 ml glycerol was added. After the addition of 375 µl DNase (10 mg/ml) the solution was incubated for 30 minutes at 37°C. Cell debris was removed by centrifugation at 1880xg for 30 minutes at RT without brake. The supernatant was subsequently purified from proteins by extraction with freon (3x). The cleared supernatant was loaded on a 1M Tris/HCl buffered caesiumchloride blockgradient (range: 1.2/1.4 gr/ml) and centrifuged at 21000 rpm for 2.5 hours at 10°C. The virus band is isolated after which a second purification using a 1M Tris/HCl buffered continues gradient of 1.33 gr/ml of caesiumchloride was performed. The virus was then centrifuged for 17 hours at 55000 rpm at 10°C. The virus band is isolated and sucrose (50 % w/v) is added to a final concentration of 1%. Excess caesiumchloride is removed by dialysis (three times 1 hr at RT) in dialysis slides (Slide-a-lizer, cut off 10000 kDa, Pierce, USA) against 1.5 ltr PBS supplemented with $CaCl_2$ (0.9 mM), $MgCl_2$ (0.5mM) and an increasing concentration of sucrose (1, 2, 5%). After dialysis, the virus is removed from the slide-a-lizer after which it is aliquoted in portions of 25 and 100 µl upon which the virus is stored at -85°C.

To determine the number of virus particles per ml, 50 µl of the virus batch is run on an high pressure liquid chromatograph (HPLC) as described by Shabram et al (1997). The virus titers of the chimaeric viruses were found to be in the same range as the Ad5.Luc virus batch (Ad5.Luc: $2.2x10^{11}$ vp/ml Ad5.Lucfib12: $1.3x10^{11}$ vp/ml; Ad5.Lucfib16: $3.1x10^{12}$ vp/ml; Ad5.Lucfib28: $5.4x10^{10}$ vp/ml; Ad5.Lucfib40-L: $1.6x10^{12}$ vp/ml).

Example 2: Biodistribution of chimaeric viruses after intravenous tail vein injection of rats.

To investigate the biodistribution of the chimaeric adenoviruses carrying fiber 12, 16, 28, or 40-L, $1x10^{10}$ particles of each of the generated virus batches were diluted to 1 ml with PBS and the virus was injected in the tail vein of adult male Wag/Rij rats (3 rats/virus). As a control, Ad5 carrying the luciferase transgene was used. Forty-eight hours after the administration of the virus, the rats were sacrificed after which the liver, spleen, lung, kidney, heart and brain were dissected. These organs were subsequently mixed with 1 ml of lysis buffer (1% Triton X-100 in PBS) and minced for 30 seconds to obtain a protein lysate. The protein lysate was tested for luciferase activity and the protein concentration was determined. The results, shown in Table II, demonstrate that in contrast to the adenovirus serotype 5 control, none of the fiber chimaeras are targeted specifically to the liver or to the spleen. This experiment shows that it is possible to circumvent the uptake of adenoviruses by the liver by making use of fibers of other serotypes. It also shows that the uptake by the liver is not correlated with the length of the fiber shaft, or determined solely by the ability of fiber knob to bind to CAR. The fibers have different shaft lengths and, except fiber 16, are derived from subgroups known to have a fiber that can bind CAR (Roelvink et al, 1998).

Example 3: Chimaeric viruses display differences in endothelial and smooth muscle cell transduction

Infection of Human umbilical vein endothelial cells

In a first set of experiments $4x10^4$ human umbilical vein endothelial (HUVEC) cells (a pool from 4 different individuals) were seeded in each well of 24-wells plate. The next day cells were washed with PBS and various amounts of

virus (50, 250, 1000, 2500, 5000, and 10000 particles/cell) were added in 200 µl of DMEM supplemented with 2% FCS. Each of the viruses Ad5.luc, Ad5.Lucfib12, Ad5.Lucfib16, Ad5.Lucfib28 and Ad5.Lucfib40-L were plated in triplicate. Two hours after addition of the virus the medium was replaced by normal medium. Forty-eight hours later cells were washed and lysed by the addition of 100 µl lysis buffer (1% Triton X-100 in PBS). Figure 7 shows luciferase activity expressed as RLU/µg protein after infection. Marked differences in infection efficiency of HUVEC cells were found. Chimaeric viruses with fiber 12 or 28 are unable to infect HUVEC cells. Ad5.Lucfib40-L infects HUVECs with similar efficiency as the control Ad5.Luc virus, whereas Ad5.Lucfib16 is able to infect HUVECs significantly better.

Infection of smooth muscle cells

To compare infection efficiency of the chimaeric viruses in human umbilical vein derived smooth muscle cells (HUVsmc) $4x10^4$ HUVsmc were seeded in wells of 24-well plates and infected as described above for the endothelial cells but using increasing MOI: 50, 250, 1250, 2500, and 5000 virus particles/cell. Figure 8 shows the luciferase activity (RLU)/µg protein assayed 48 hrs after infection. The results show that Ad5.Lucfib12 and Ad5.Lucfib28 are also unable to infect HUVsmc cells, that 40-L infects HUVsmc with similar efficiency as the control Ad5 virus, and that Ad5.Lucfib16 chimaeric viruses infect HUVsmc extremely efficient (~80x better).

From the above described results it is clear that the chimaeric adenovirus with the shaft and knob from fiber 16 is well suited to infect endothelial cells and smooth muscle cells. Thus by changing the fiber protein on Ad5 viruses we are able to increase infection of cells that are poorly

infected by Ad5. The difference between Ad5.Lucfib16 and Ad5Luc, although significant on both cell types, is less striking on endothelial cells as compared to smooth muscle cells. This may reflect differences in receptor expression. For example HUVsmc express significantly more $\alpha_v\beta_5$ integrins than HUVEC cells (see below). Alternatively this difference may be due to differences in expression of the receptor of fiber 16. Ad5.Lucfib16 viruses infect umbilical vein smooth muscle cells approximately 7x better than endothelial cells whereas in case of Ad5Luc viruses endothelial cells are better infected than SMC. To test whether Ad5 infection correlated with receptor expression of these cells the presence of CAR and α_v-integrin was assayed on a flow cytometer. For this purpose $1x10^5$ HUVEC cells or HUVsmc were washed once with PBS/0.5% BSA. Cells were then pelleted by centrifugation for 5 minutes at 1750 rpm at RT. Subsequently, 10 µl of a 100 times diluted $\alpha_v\beta3$ antibody (Mab 1961, Brunswick Chemie, Amsterdam, The Netherlands), a 100 times diluted antibody $\alpha_v\beta5$ (antibody (Mab 1976, Brunswick chemie, Amsterdam, The Netherlands), or 2000 times diluted CAR antibody (RmcB, a kind gift of Dr. Bergelson, Harvard Medical School, Boston, USA; Hsu et al, 1988) was added to the cell pellet after which the cells were incubated for 30 minutes at 4°C in a dark environment. After this incubation, cells were washed twice with PBS/0.5% BSA and again pelleted by centrifugation for 5 minutes at 1750 rpm room temperature. To label the cells, 10 µl of rat anti mouse IgG1 labelled with phycoerythrine (PE) was added to the cell pellet upon which the cells were again incubated for 30 minutes at 4°C in a dark environment. Finally the cells were washed twice with PBS/0.5% BSA and analysed on a flow cytometer. The results of these experiments are shown in Table III.

From the results it can be concluded that HUVsmc do not express detectable levels of CAR confirming that these cells

are difficult to transduce with an adenovirus which enters the cells via the CAR receptor.

As a control for the experiments performed on endothelial cells and smooth muscle cells, A549 cells were infected to establish whether an equal amount of virus particles of the different chimaeric adenoviruses show significant differences in transgene expression on cell lines that are easily infected by adenovirus. This is to investigate whether the observed differences in infection efficiency on the endothelial and smooth muscle cells are cell type specific. For this purpose, 10^5 A549 cells were seeded in 24-well plates in a volume of 200 µl. Two hours after seeding the medium was replaced by medium containing different amounts of particles of either fiber chimera 5, 12, 16, or 40-L (MOI = 0, 5, 10, 25, 100, 500). Twenty-four hours after the addition of virus, the cells were washed once with PBS after which the cells were lysed by the addition of 100 µl lysis buffer to each well (1% Triton X-100 in PBS) after which transgene expression (luciferase activity) and the protein concentration was determined. The data, shown in table IV demonstrate that Ad5.Luc viruses infect A549 cells most efficient while the infection efficiency of Ad5.Lucfib16 and Ad5.Lucfib40-L is a few times lower. This means that the efficient infection of endothelial cells and especially smooth muscle cells is due to differences in binding of the virus to these cells and not to the amount of virus or quality of the viruses used.

TABLES

Table I: Oligonucleotides and degenerate oligonucleotides used for the amplification of DNA encoding fiber protein derived from alternative adenovirus serotypes. (Bold letters represent NdeI restriction site (A-E), NsiI restriction site (1-7, 8), or PacI restriction site (7).

Serotype	Tail oligonucleotide	Knob oligonucleotide
4	A	1
8	B	2
9	B	2
12	E	3
16	C	4
19p	B	2
28	B	2
32	B	2
36	B	2
37	B	2
40-1	D	5
40-2	D	6
41-s	D	5
41-l	D	7
49	B	2
50	B	2
51	C	8

A: 5'- CCC GTG TAT **CCA TAT** GAT GCA GAC AAC GAC CGA CC- 3'

B: 5'- CCC GTC TAC **CCA TAT** GGC TAC GCG CGG- 3'

C: 5'- CCK GTS TAC **CCA TAT** GAA GAT GAA AGC- 3'

D: 5'- CCC GTC TAC **CCA TAT** GAC ACC TYC TCA ACT C- 3'

E: 5'- CCC GTT TAC **CCA TAT** GAC CCA TTT GAC ACA TCA GAC- 3'

1: 5''- CCG **ATG CAT** TTA TTG TTG GGC TAT ATA GGA - 3'

2: 5'- CCG **ATG CAT** TYA TTC TTG GGC RAT ATA GGA - 3'

3: 5'- CCG **ATG CAT** TTA TTC TTG GGR AAT GTA WGA AAA GGA - 3'

4: 5'- CCG **ATG CAT** TCA GTC ATC TTC TCT GAT ATA - 3'

5: 5'- CCG **ATG CAT** TTA TTG TTC AGT TAT GTA GCA - 3'

6: 5'- GCC **ATG CAT** TTA TTG TTC TGT TAC ATA AGA - 3'

7: 5' - CCG **TTA ATT AAG** CCC TTA TTG TTC TGT TAC ATA AGA A - 3'

8: 5'- CCG **ATG CAT** TCA GTC ATC YTC TWT AAT ATA - 3'

Table II:

Biodistribution of chimaeric adenovirus upon intravenous tail vein injection. Values represent luciferase activity expressed as relative light units/µg protein. Values in the brain are considered background.

Organ	Ad5.Luc	Ad5.Luc-fib 12	Ad5.Luc-fib16	Ad5.Luc-fib28	Ad5.Luc-fib40-L
Liver	740045	458	8844	419	2033
Spleen	105432	931	3442	592	16107
Lung	428	315	334	316	424
Kidney	254	142	190	209	224
Heart	474	473	276	304	302
Brain	291	318	294	323	257

Table III:

Expression of CAR and integrins on the cell surface of endothelial cells and smooth muscle cells. 70%: Cells harvested for FACS analysis at a cell density of 70% confluency. 100%: Cells harvested for FACS analysis at a cell density of 100% confluency. PER.C6 cells were taken as a control for antibody staining. Values represent percentages of cells that express CAR or either one of the integrins at levels above background. For background control HUVECs or HUVsmc were incubated only with the secondary, rat-anti-mouse IgG1-PE labelled antibody.

Cell line	$\alpha_v\beta3$	$\alpha_v\beta5$	CAR
HUVEC 70%	98.3%	18.9%	18.1%
HUVEC 100%	97.2%	10.5%	7.2%
HUVsmc 70%	95.5%	76.6%	0.3%
HUVsmc 100%	92.2%	66.5%	0.3%
PER.C6	7.8%	16.8%	99.6%

Table IV:

Determination of transgene expression (luciferase activity) per µg of total cellular protein after infection of A549 cells.

MOI (VP/cell)	Ad5.Luc	Ad5.Luc-fib12	Ad5.Luc-fib16	Ad5.Luc-fib40-L
0	0	0	0	0
5	1025	46	661	443
10	1982	183	1704	843
25	4840	200	3274	2614
100	21875	1216	13432	11907
500	203834	3296	93163	71433

REFERENCES

Arnberg N., Mei Y. and Wadell G., 1997. Fiber genes of adenoviruses with tropism for the eye and the genital tract. Virology 227: 239-244.

Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L. and Finberg, R.W. (1997) Isolation of a common receptor for coxsackie B virus and adenoviruses 2 and 5. Science 275: 1320-1323.

Bout A. (1997) Gene therapy, p. 167-182. In: D.J.A. Crommelin and R.D. Sindelar (ed.), Pharmaceutical Biotechnology , Harwood Academic Publishers.

Bout, A. (1996) Prospects for human gene therapy. Eur. J. Drug Met. and Pharma. 2, 175-179.

Blaese, M., Blankenstein, T., Brenner, M., Cohen-Hagenauer, O., Gansbacher, B., Russel, S., Sorrentino, B. and Velu, T. (1995) Cancer Gene Ther. 2: 291-297.

Brody, S.L. and Crystal, R.G. (1994) Adenovirus mediated in vivo gene transfer. Ann. N. Y. Acad. Sci. 716:90-101.

Carter, A.J., Laird, J.R., Farb, A., Kufs, W., Wortham, D.C. and Virmani, R. (1994) Morphologic characteristics of lesion formation and time course of smooth muscle cell proliferation in a porcine proliferative restenosis model. J. Am. Coll. Cardiol. 24: 1398-1405.

Chroboczek J., Ruigrok R.W.H., and Cusack S., 1995. Adenovirus fiber, p. 163-200. In: W. Doerfler and P. Bohm (ed.), The molecular repertoire of adenoviruses, I. Springer-Verlag, Berlin.

Defer C., Belin M., Caillet-Boudin M. and Boulanger P., 1990. Human adenovirus-host cell interactions; comparative study with members of subgroup B and C. Journal of Virology 64 (8): 3661-3673.

Fallaux, F.J, Bout, A, van der Velde, I et al. New helper cells and matched E1-deleted adenovirus vectors prevent generation of replication competent adenoviruses. Human Gene Therapy, 9 (1998), p1909-1917.

Francki, R.I.B., Fauquet, C.M., Knudson, D.L. and Brown, F. (1991) Classification and nomenclature of viruses. Fifth report of the international Committee on taxonomy of viruses. Arch. Virol. Suppl. 2: 140-144.

Gall J., Kass-Eisler A., Leinwand L. and Falck-Pedersen E. (1996) Adenovirus type 5 and 7 capsid chimera: fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. Journal of Virology 70 (4): 2116-2123.

Greber, U.F., Willets, M., Webster, P., and Helenius, A. (1993). Stepwise dismanteling of adenovirus 2 during entry into cells. Cell 75: 477-486.

Hynes, R.O. (1992) Integrins: versatility, modulation and signalling in cell adhesion. Cell 69: 11-25.

Herz, J. and Gerard, R.D. (1993) Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearence in normal mice. Proc. Natl. Acad. Sci. U.S.A. 96: 2812-2816.

Hierholzer, J.C. (1992) Adenovirus in the immunocompromised host. Clin. Microbiol Rev. 5, 262-274.

Hierholzer, J.C., Wigand, R., Anderson, L.J., Adrian, T., and Gold, J.W.M. (1988) Adenoviruses from patients with AIDS: a plethora of serotypes and a description of five new serotypes of subgenus D (types 43-47). J. Infect. Dis. 158, 804-813.

Hong, S.S., Karayan, L., Tournier, J., Curiel, D.T. and Boulanger, P.A. (1997) Adenovirus type 5 fiber knob binds to MHC class I α2 domain at the surface of human epithelial and B lymphoblastoid cells. EMBO J. 16: 2294-2306.

Hsu, K.H., Lonberg-Holm, K., Alstein, B. and Crowell, R.L. (1988)
A monoclonal antibody specific for the cellular receptor for the group B coxsackieviruses. J. Virol 62(5): 1647-1652.

Huard, J., Lochmuller, H., Acsadi, G., Jani, A., Massie, B. and Karpati, G. (1995) The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. Gene Ther. 2: 107-115.

Ishibashi, M. and Yasue, H. (1984) The adenoviruses, H.S. Ginsberg, ed., Plenum Press, Londen, New York. Chapter 12, 497-561.

Kass-Eisler, A., Falck-Pederson, E., Elfenbein, D.H., Alvira, M., Buttrick, P.M. and Leinwand, L.A. (1994) The impact of developmental stage, route of administration and the immune system on adenovirus-mediated gene transfer. Gene Ther. 1: 395-402.

Khoo, S.H., Bailey, A.S., De Jong, J.C., and Mandal, B.K. (1995). Adenovirus infections in human immunodeficiency virus-positive patients: Clinical features and molecular epidemiology. J. Infect. Dis 172, 629-637

Kidd, A.H., Chroboczek, J., Cusack, S., and Ruigrok, R.W. (1993) Adenovirus type 40 virions contain two distinct fibers. Virology 192, 73-84.

Krasnykh V.N., Mikheeva G.V., Douglas J.T. and Curiel D.T. (1996) Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. J. Virol. 70(10): 6839-6846.

Krasnykh V.N., Dmitriev I., Mikheeva G., Miller C.R., Belousova N. and Curiel D.T. (1998) Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. J. Virol. 72(3): 1844-1852.

Law, L., Chillon, M., Bosch, A., Armentano, D., Welsh, M.J. and Davidson, B.L. (1998) Infection of primary CNS cells by different adenoviral serotypes: Searching for a more efficient vector. Abstract 1st Annual Meeting American Society of Gene Therapy, Seattle, Washington.

Leppard, K.N. (1997) E4 gene function in adenovirus, adenovirus vector and adeno-associated virus infections. J. Gen. Virol. 78: 2131-2138.

Lloyd Jones, D.M. and Bloch, K.D. (1996) The vascular biology of nitric oxide and its role in atherogenesis. Annu. Rev. Med. 47: 365-375.

Morgan, C., Rozenkrantz, H.S., and Mednis, B. (1969) Structure and development of viruses as observed in the electron microscope.X. Entry and uncoating of adenovirus. J. Virol 4, 777-796.

Roelvink, P.W., Kovesdi, I. and Wickham, T.J. (1996) Comparative analysis of adenovirus fiber-cell interaction: Adenovirus type 2 (Ad2) and Ad9 utilize the same cellular

fiber receptor but use different binding stratagies for attachemnt. J. Virol. 70: 7614-7621.

Roelvink, P.W., Lizonova, A., Lee, J.G.M., Li, Y., Bergelson, J.M., Finberg, R.W., Brough, D.E., Kovesdi, I. and Wickham, T.J. (1998) The coxsackie-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. J. Virol. 72: 7909-7915.

Rogers, B.E., Douglas J.T., Ahlem, C., Buchsbaum, D.J., Frincke, J. and Curiel, D.T. (1997) Use of a novel cross-linking method to modify adenovirus tropism. Gene Ther.4: 1387-1392.

Schulick, A.H., Vassalli, G., Dunn, P.F., Dong, G., Rade, J.J., Zamarron, C. and Dichek, D.A. (1997). Established immunity precludes adenovirus-mediated gene transfer in rat carotid arteries.

Schnurr, D and Dondero, M.E. (1993) Two new candidate adenovirus serotypes. Intervirol. 36, 79-83.

Schwartz, R.S., Edwards, W.D., Huber, K.C., Antoniudes, L.C. Bailey, K.R., Camrud, A.R., Jorgenson, M.A. and Holmes, D.R. Jr. (1993) Coronary restenosis: Prospects for solution and new perspectives from a porcine model. Mayo Clin. Proc. 68: 54-62.

Shi, Y., Pieniek, M., Fard, A., O'Brien, J., Mannion, J.D. and Zalewski, A. (1996) Adventitial remodelling after coronary arterial injury. Circulation 93: 340-348.

Shabram, P.W., Giroux, D.D., Goudreau, A.M., Gregory, R.J., Horn, M.T., Huyghe, B.G., Liu, X., Nunnally, M.H., Sugarman, B.J. and Sutjipto, S. (1997) Analytical anion-exchange HPLC

of recombinant type-5 adenoviral particles. Hum. Gene Ther. 8(4): 453-465.

Signas, G., Akusjarvi, G., and Petterson, U. (1985). Adenovirus 3 fiberpolypeptide gene: Complications for the structure of the fiber protein. J. Virol. 53, 672-678.

Stevenson S.C., Rollence M., White B., Weaver L. and McClelland A., (1995) Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. J. Virol 69(5): 2850-2857.

Stevenson S.C., Rollence M., Marshall-Neff J. and McClelland A. (1997) Selective targeting of human cells by a chimaeric adenovirus vector containing a modified fiber protein. J. Virology 71(6): 4782-4790.

Stouten, P.W.F., Sander, C., Ruigrok, R.W.H., and Cusack, S. (1992) New triple helical model for the shaft of the adenovirus fiber. J. Mol. Biol. 226, 1073-1084.

Svensson, V. and Persson, R. (1984). Entry of adenovirus 2 into Hela cells. J. Virol. 51, 687-694.

Van der Vliet, P.C. (1995) Adenovirus DNA replication In: W. Doerfler and P. Böhm (eds.), The molecular repertoire of adenoviruses II. Springer-Verlag, Berlin.

Varga, M.J., Weibull, C., and Everitt, E. (1991). Infectious entry pathway of adenovirus type 2. J. Virol 65, 6061-6070.

Varenne, O., Pislaru, S., Gillijns, H., Van Pelt, N., Gerard, R.D., Zoldhelyi, P., Van de Werf, F., Collen, D. and Janssens, S.P. (1998) Local adenovirus-mediated transfer of human endothelial nitric oxide synthetase reduces luminal narrowing after coronary angioplasty in pigs. Circulation 98: 919-926.

Wickham T.J., Carrion M.E. and Kovesdi I., 1995. Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs. Gene Therapy **2**: 750-756.

Wickham T.J., Segal, D.M., Roelvink, P.W., Carrion M.E., Lizonova, A., Lee, G-M., and Kovesdi, I. (1996) Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. J. Virol. 70 (10), 6831-6838.

Wickham, T.J., Mathias, P., Cherish, D.A., and Nemerow, G.R. (1993) Integrins avb3 and avb5 promote adenovirus internalisation but not virus attachment. Cell 73, 309-319.

Wold, W.S., Tollefson, A.E. and Hermiston, T.W. (1995) E3 transcription unit of adenovirus. In: W. Doerfler and P. Böhm (eds.), The molecular repertoire of adenoviruses I. Springer-Verlag, Berlin.

Zabner, J., Armentano, D., Chillon, M., Wadsworth, S.C. and Welsh, M.J. (1998) Type 17 fiber enhances gene transfer Abstract 1st Annual Meeting American Society of Gene Therapy, Seattle, Washington.

CLAIMS

1. A gene delivery vehicle having been provided with at least a tissue tropism for smooth muscle cells and/or endothelial cells.

2. A gene delivery vehicle having been deprived of at least a tissue tropism for liver cells.

3. A vehicle according to claim 1 wherein said vehicle has been deprived of at least a tissue tropism for liver cells.

4. A vehicle according to anyone of the claims 1-3, wherein said tissue tropism is being provided by a virus capsid.

5. A vehicle according to claim 4, wherein said capsid comprises protein fragments from at least two different viruses.

6. A vehicle according to claim 5, wherein at least one of said viruses is an adenovirus.

7. A vehicle according to claim 5 or claim 6, wherein at least one of said viruses is an adenovirus of subgroup B.

8. A vehicle according to anyone of the claims 5-7, wherein at least one of said protein fragments comprises a tissue tropism determining fragment of a fiber protein derived from a subgroup B adenovirus.

9. A vehicle according to anyone of the claim 7 or claim 8, wherein said subgroup B adenovirus is adenovirus 16.

10. A vehicle according to claim 7-9, wherein protein fragments not derived from an adenovirus of subgroup B are derived from an adenovirus of subgroup C, preferably of adenovirus 5.

11. A vehicle according to anyone of the claims 1-10 comprising a nucleic acid derived from an adenovirus.

12. A vehicle according to anyone of the claims 1-11, comprising a nucleic acid derived from at least two different adenoviruses.

13. A vehicle according to claim 11 or claim 12, wherein said nucleic acid comprises at least one sequence encoding a fiber protein comprising at least a tissue tropism determining fragment of a subgroup B adenovirus fiber protein, preferably of adenovirus 16.

14. A vehicle according anyone of the claims 10-13, wherein said adenovirus nucleic acid is modified such that the capacity of said adenovirus nucleic acid to replicate in a target cell has been reduced or disabled.

15. A vehicle according to anyone of the claims 11-14, wherein said adenovirus nucleic acid is modified such that the capacity of a host immune system to mount an immune response against adenovirus proteins encoded by said adenovirus nucleic acid has been reduced or disabled.

16. A vehicle according to anyone of the claims 1-15, comprising a minimal adenovirus vector or an Ad/AAV chimaeric vector.

17. A vehicle according to anyone of the claims 1-16, further comprising at least one non-adenovirus nucleic acid.

18. A vehicle according to claim 17 wherein at least one of said non-adenovirus nucleic acids is a gene selected from the group of genes encoding: an apolipoprotein, a nitric oxide synthase, a herpes simplex virus thymidine kinase, an interleukin-3, an interleukin-1α, an (anti)angiogenesis protein such as angiostatin, an anti-proliferation protein, a smooth muscle cell anti-migration protein, a vascular endothelial growth factor (VGEF), a basic fibroblast growth factor, a hypoxia inducible factor 1α (HIF-1α) or a PAI-1.

19. A cell for the production of a vector according to anyone of the claims 1-18, comprising means for the assembly of said vectors wherein said means includes a means for the production of an adenovirus fiber protein, wherein said fiber protein comprises at least a tissue tropism determining fragment of a subgroup B adenovirus fiber protein.

20. A cell according to claim 19, wherein said cell is or is derived from a PER.C6 cell (ECACC deposit number 96022940).

21. The use of a vehicle according to anyone of the claims 1-18 as a pharmaceutical.

22. The use of claim 21 for the treatment of cardiovascular disease.

23. The use of claim 21 for the treatment of a disease, treatable by transfer of a therapeutic nucleic acid to smooth muscle cells and/or endothelial cells.

24. An adenovirus capsid with or provided with a tissue tropism for smooth muscle cells and/or endothelial cells wherein said capsid preferably comprises proteins from at least two different adenoviruses and wherein at least a tissue tropism determining fragment of a fiber protein is derived from a subgroup B adenovirus, preferably of adenovirus 16.

25. An adenovirus capsid having been deprived of a tissue tropism for liver cells wherein said capsid preferably comprises proteins from at least two different adenoviruses and wherein at least a tissue tropism determining fragment of a fiber protein is derived from a subgroup B adenovirus, preferably of adenovirus 16.

26. The use of an adenovirus capsid according to claim 24 and/or claim 25, for the delivery of nucleic acid to smooth muscle cells and/or endothelial cells.

27. The use of an adenovirus capsid according to claim 26, in a medicament for the treatment of a disease.

28. Construct pBr/Ad.BamRΔFib, comprising adenovirus 5 sequences 21562-31094 and 32794-35938.

29. Construct pBr/AdBamRfib16, comprising adenovirus 5 sequences 21562-31094 and 32794-35938, further comprising an adenovirus 16 gene encoding fiber protein.

30. Construct pBr/AdBamR.pac/fib16, comprising adenovirus 5 sequences 21562-31094 and 32794-35938, further comprising an adenovirus 16 gene encoding fiber protein, and further comprising a unique PacI-site in the proximity of the adenovirus 5 right terminal repeat, in the non-adenovirus sequence backbone of said construct.

31. Construct pWE/Ad.AflIIrITRfib16, comprising adenovirus 5 sequences 3534-31094 and 32794-35938, further comprising an adenovirus 16 gene encoding fiber protein.

32. Construct pWE/Ad.AflIIrITRDE2Afib16, comprising adenovirus 5 sequences 3534-22443, 24033-31094 and 32794-35938, further comprising an adenovirus 16 gene encoding fiber protein.

33. The use of a construct according to anyone of the claims 28-32 for the generation of a vehicle according to anyone of the claims 1-18 or an adenovirus capsid according to claim 24 or claim 25.

34. The production of a vehicle according to anyone of the claims 1-18 or a adenovirus capsid according to claim 24 or claim 25.

35. The use of a vehicle according to anyone of the claims 1-18 for the generation a gene library.

36. The use of a fiber protein of adenovirus 16 for the delivery of nucleic acid to smooth muscle cells and/or endothelial cells.

37. The use of a fiber protein of adenovirus 16 in an adenovirus capsid for depriving said capsid of a tissue tropism for liver cells.

Fig 1

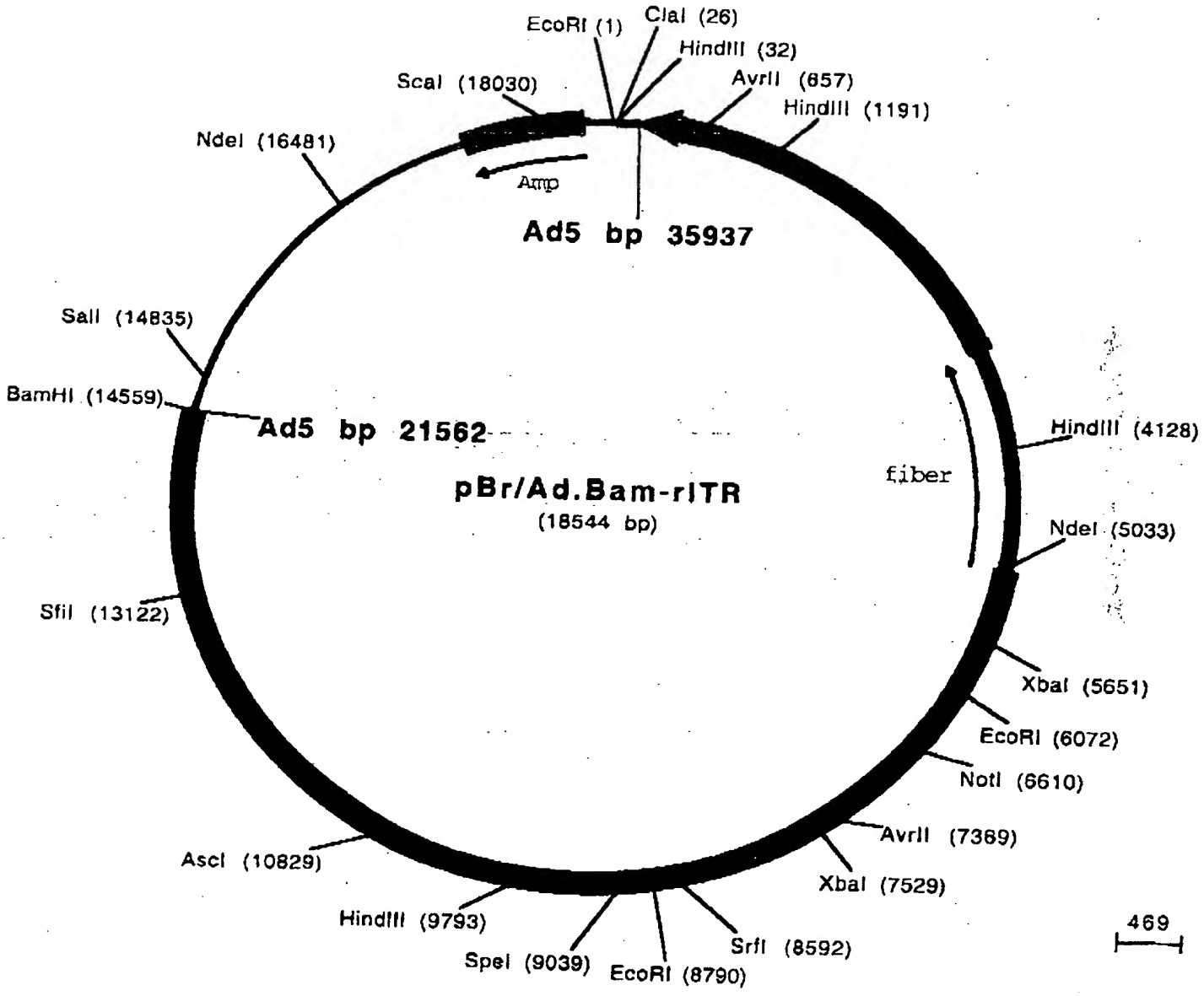
EcoRI (1)
ClaI (26)
HindIII (32)
AvrII (857)
HindIII (1191)
ScaI (18030)
NdeI (16481)
Amp
Ad5 bp 35937
SalI (14835)
BamHI (14559)
Ad5 bp 21562
pBr/Ad.Bam-rITR
(18544 bp)
fiber
HindIII (4128)
NdeI (5033)
SfiI (13122)
XbaI (5651)
EcoRI (6072)
NotI (6610)
AvrII (7369)
AscI (10829)
XbaI (7529)
HindIII (9793)
SrfI (8592)
SpeI (9039)
EcoRI (8790)
469

Figure 2

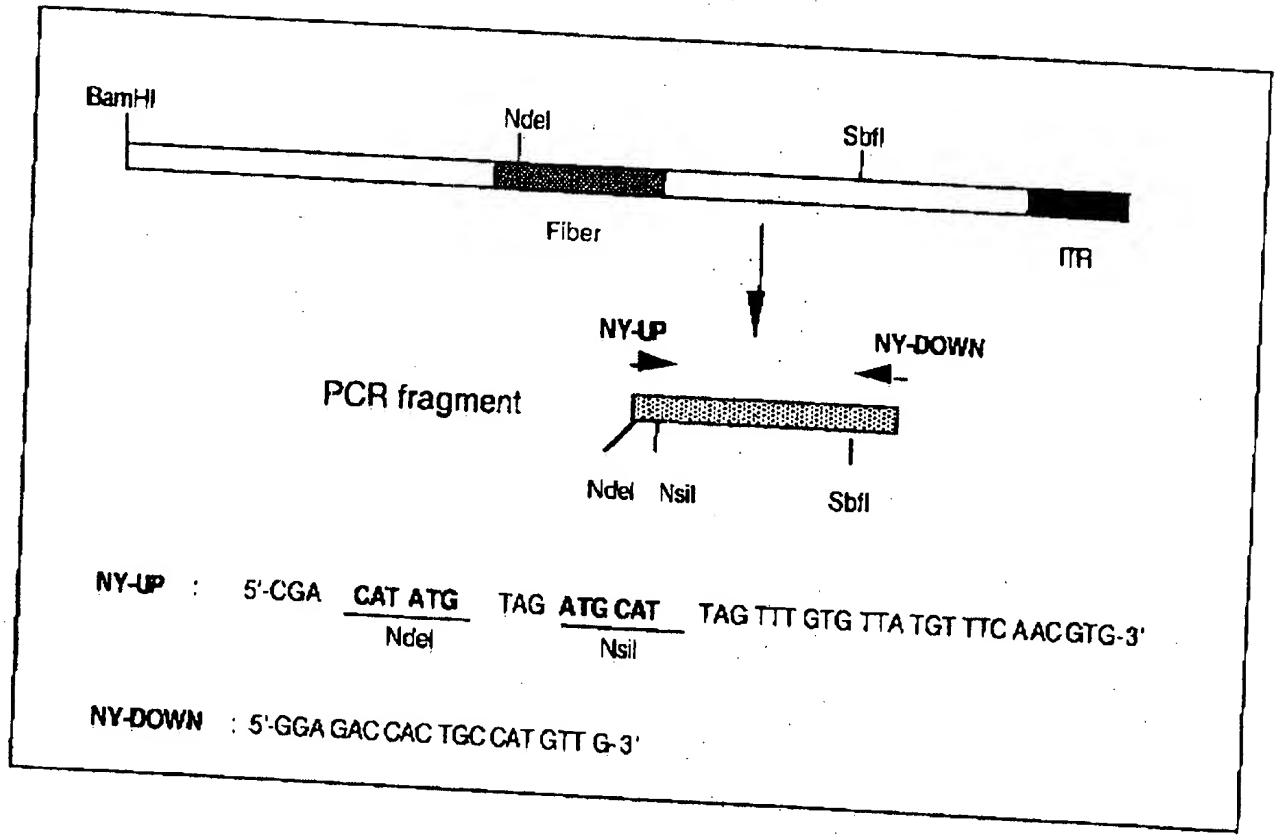
BamHI
NdeI
SbfI
Fiber
ITR
NY-UP
NY-DOWN
PCR fragment
NdeI
NsiI
SbfI
NY-UP : 5'-CGA CAT ATG TAG ATG CAT TAG TTT GTG TTA TGT TTC AAC GTG-3'
NdeI
NsiI
NY-DOWN : 5'-GGA GAC CAC TGC CAT GTT G-3'

fig 3

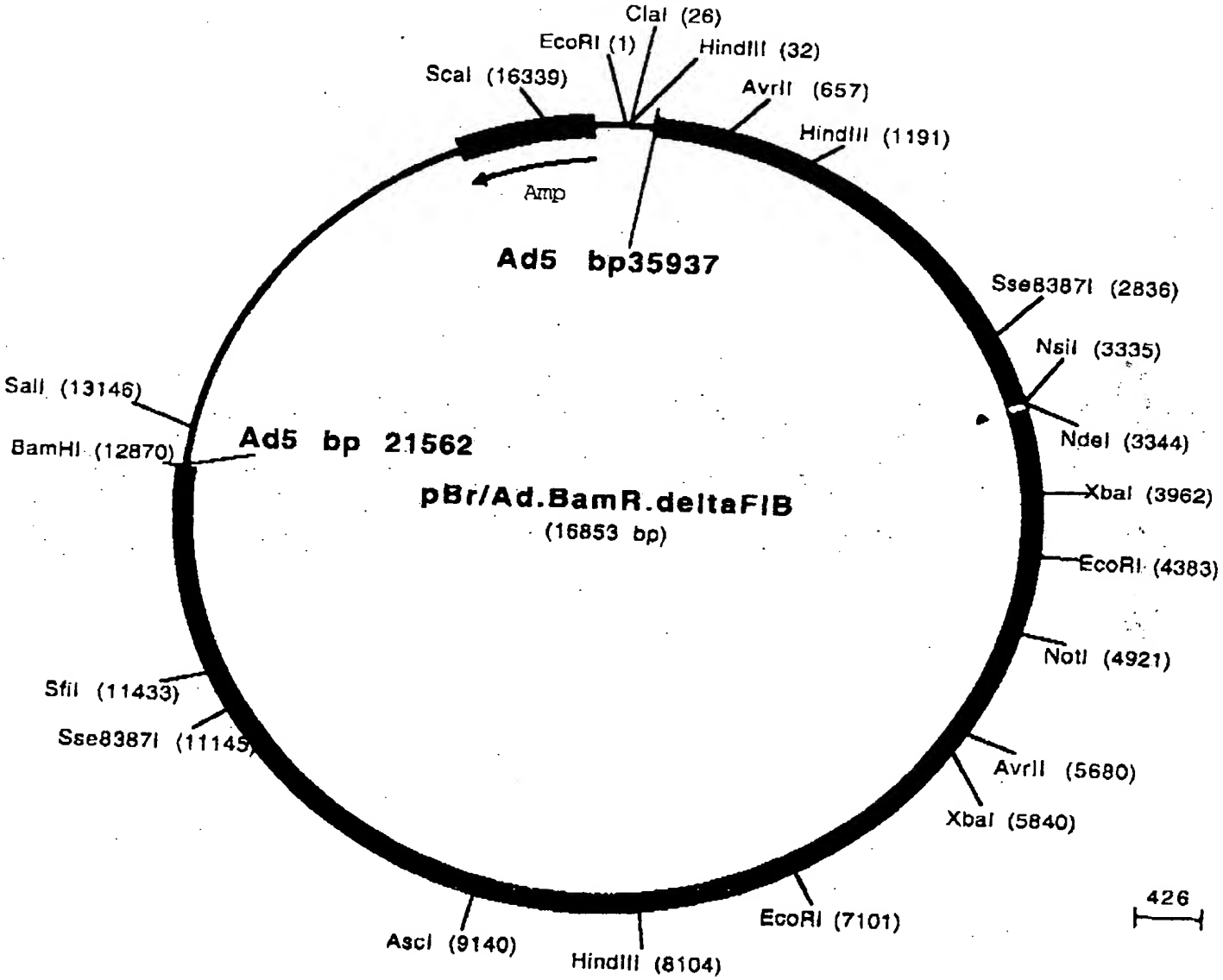
ClaI (26)
EcoRI (1)
HindIII (32)
ScaI (16339)
AvrII (657)
HindIII (1191)
Amp
Ad5 bp35937
Sse8387I (2836)
NsiI (3335)
SalI (13146)
NdeI (3344)
BamHI (12870)
Ad5 bp 21562
XbaI (3962)
pBr/Ad.BamR.deltaFIB
(16853 bp)
EcoRI (4383)
NotI (4921)
SfiI (11433)
Sse8387I (11145)
AvrII (5680)
XbaI (5840)
426
EcoRI (7101)
AscI (9140)
HindIII (8104)

Figure 4A: Sequence of Ad5 fiber

ATGAAGCGCGCAAGACCGTCTGAAGATACCTTCAACCCCGTGTATCCATATGACACGGAAACCGGTC
CTCCAACTGTGCCTTTTCTTACTCCTCCCTTTGTATCCCCCAATGGGTTTCAAGAGAGTCCCCCTGG
GGTACTCTCTTTGCGCCTATCCGAACCTCTAGTTACCTCCAATGGCATGCTTGCGCTCAAAATGGGC
AACGGCCTCTCTCTGGACGAGGCCGGCAACCTTACCTCCCAAAATGTAACCACTGTGAGCCCACCTC
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AACGGTACACAGGAAACAGGAGACACAACTCCAAGTGCATACTCTATGTCATTTTCATGGGACTGGT
CTGGCCACAACTACATTAATGAAATATTTGCCACATCCTCTTACACTTTTTCATACATTGCCCAAGA
ATAA

Figure 4B: Sequence of Ad5/fib12 chimeric fiber

ATGAAGCGCGCAAGACCGTCTGAAGATACCTTCAACCCCGTGTATCCATATGACCCATTTGACACAT
CAGACGTACCCTTTGTTACACCCCCTTTTACTTCTTCCAATGGTCTTCAAGAAAAACCACCAGGTGT
ATTAGCACTTAATTACAAAGACCCCATTGTAACTGAAAATGGAACCCTTACACTCAAGCTAGGGGAC
GGAATAAAACTTAATGCCCAAGGTCAACTTACAGCTAGTAATAATATCAATGTTTTGGAGCCCCTTA
CCAACACCTCACAAGGTCTTAAACTTTCTTGGAGCGCCCCCCTAGCAGTAAAGGCTAGTGCCCTCAC
ACTTAACACAAGAGCGCCCTTAACCACAACGGATGAAAGCTTAGCCTTAATAACCGCCCCTCCCATT
ACAGTAGAGTCTTCGCGTTTGGGCTTGGCCACCATAGCCCCTCTAAGCTTAGATGGAGGTGGAAACC
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CTTAGTTGTAAATTCTAGCGGTGCCCTATCTGTTGCTACTGCAGACCCCATAAGTGTTCGCAACAAC
GCTCTTACCCTACCTACGGCAGATCCGTTAATGGTGAGCTCCGATGGGTTGGGAATAAGTGTCACTA
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GGGTCATGAAAGTAAACGTTGCCGGGGGAATGAGAACTTCTGGCGGTAGAATAATTTTAGATGTTAA
TTATCCCTTTGATGCGAGCAATAACCTGTCCTTAAGACGGGGATTGGGACTAATTTATAACCAATCT
ACAAACTGGAACTTAACAACTGATATTAGTACCGAAAAAGGTTTAATGTTTAGTGGCAATCAAATAG
CTCTTAATGCAGGTCAGGGGCTTACATTTAATAATGGCCAACTTAGGGTTAAGTTGGGAGCTGGACT
TATTTTTGATTCAAACAATAACATTGCCTTAGGCAGCAGCAGCAACACTCCATACGACCCTCTGACA
CTGTGGACAACTCCTGACCCACCACCAAACTGCAGCCTCATACAAGAGCTAGATGCAAAACTCACCC
TGTGCTTAACAAAAAACGGATCTATTGTTAATGGCATTGTAAGTTTAGTGGGTGTTAAGGGTAATCT
CCTAAATATCCAAAGTACTACTACCACTGTAGGAGTGCATTTAGTGTTTGATGAACAGGGAAGATTA
ATCACATCAACCCCTACTGCCCTGGTTCCCCAAGCTTCGTGGGGATATAGACAAGGCCAATCAGTGT
CTACCAATACTGTTACCAATGGTCTAGGTTTTATGCCTAATGTGAGTGCTTACCCTAGACCAAATGC
CAGTGAGGCTAAAAGCCAAATGGTAAGTCTCACGTACTTACAGGGAGATACATCTAAACCTATAACA
ATGAAAGTTGCATTTAATGGCATTACGTCGCTAAATGGATACTCTTTAACATTCATGTGGTCAGGTC
TATCAAACTATATAAATCAGCCTTTCTCTACACCATCCTGCTCCTTNTCTTACATTGCCCAAGAA**TA**
AATGCATTAG

Figure 4C: Sequence of Ad5/fib16 chimeric fiber

ATGAAGCGCGCAAGACCGTCTGAAGATACCTTCAACCCCGTGTATCCATATGAAGATGAAAGCAGCT
CACAACACCCCTTTATAAACCCTGGTTTCATTTCCTCAAATGGTTTTGCACAAAGCCCAGATGGAGT
TCTAACTCTTAAATGTGTTAATCCACTCACTACCGCCAGCGGACCCCTCCAACTTAAAGTTGGAAGC
AGTCTTACAGTAGATACTATCGATGGGTCTTTGGAGGAAAATATAACTGCCGAAGCGCCACTCACTA
AAACTAACCACTCCATAGGTTTATTAATAGGATCTGGCTTGCAAACAAAGGATGATAAACTTTGTTT
ATCGCTGGGAGATGGGTTGGTAACAAAGGATGATAAACTATGTTTATCGCTGGGAGATGGGTTAATA
ACAAAAAATGATGTACTATGTGCCAAACTAGGACATGGCCTTGTGTTTGACTCTTCCAATGCTATCA
CCATAGAAAACAACACCTTGTGGACAGGCGCAAAACCAAGCGCCAACTGTGTAATTAAAGAGGGAGA
AGATTCCCCAGACTGTAAGCTCACTTTAGTTCTAGTGAAGAATGGAGGACTGATAAATGGATACATA
ACATTAATGGGAGCCTCAGAATATACTAACACCTTGTTTAAAAACAATCAAGTTACAATCGATGTAA
ACCTCGCATTTGATAATACTGGCCAAATTATTACTTACCTATCATCCCTTAAAAGTAACCTGAACTT
TAAAGACAACCAAAACATGGCTACTGGAACCATAACCAGTGCCAAAGGCTTCATGCCCAGCACCACC
GCCTATCCATTTATAACATACGCCACTGAGACCCTAAATGAAGATTACATTTATGGAGAGTGTTACT
ACAAATCTACCAATGGAACTCTCTTTCCACTAAAAGTTACTGTCACACTAAACAGACGTATGTTAGC
TTCTGGAATGGCCTATGCTATGAATTTTTCATGGTCTCTAAATGCAGAGGAAGCCCCGGAAACTACC
GAAGTCACTCTCATTACCTCCCCCTTCTTTTTTTCTTATATCAGAGAAGATGAC**TGA**ATGCATTAG

Figure 4D: Sequence of Ad5/fib28 chimeric fiber

ATGTTGTTGCAGATGAAGCGCGCAAGACCGTCTGAAGATACCTTCAACCCCGTGTATCCATATGGCT
ACGCGCGGAATCAGAATATCCCCTTCCTCACTCCCCCCTTTGTTTCTTCCGATGGATTCCAAAACTT
CCCACCTGGGGTCCTGTCACTCAAACTGGCTGACCCAATCACCATCGCTAATGGGGATGTCTCACTC
AAGTTGGGAGGCGGACTGACGGTGGAAAAAGAGTCTGGAAACTTAACTGTGAACCCTAAGGCTCCCT
TGCAAGTTGCAAGTGGACAATTGGAATTAGCATATGATTCTCCATTTGATGTTAAAAACAATATGCT
TACTCTTAAAGCAGGTCACGGCTTAGCAGTTGTAACGAAAGACAATACTGATTTACAACCACTAATG
GGCACACTTGTTGTTTTAACTGGCAAAGGCATTGGCACTGGCACAAGTGCTCACGGTGGAACCATAG
ATGTGAGAATAGGAAAAAACGGAAGTCTGGCATTTGACAAAAATGGAGATTTGGTGGCCTGGGATAA
AGAAAATGACAGGCGCACTCTATGGACAACTCCAGACACATCTCCAAATTGCAAAATGAGTGAAGTC
AAAGACTCAAAGCTTACTCTTATTCTTACAAAATGCGGAAGTCAAATTCTAGGAAGTGTATCTTTGC
TTGCTGTAAAAGGAGAATATCAAAATATGACTGCCAGTACTAATAAGAATGTAAAAATAACACTGCT
ATTTGATGCTAATGGAGTCTTGTTAGAAGGATCCAGTCTTGATAAAGAGTACTGGAACTTTAGAAAC
AATGATTCTACTGTGTCTGGAAAATATGAAAATGCTGTTCCGTTCATGCCTAACATAACAGCTTATA
AACCCGTCAATTCTAAAAGCTATGCCAGAAGTCACATATTTGGAAATGTATATATTGCTGCTAAGCC
ATATAATCCAGTGGTTATTAAAATTAGCTTCAATCAAGAGACACAAAACAATTGTGTCTATTCTATA
TCATTTGACTACACTTGCTCTAAAGAGTATACAGGTATGCAATTCGATGTTACATCTTTCACCTTCT
CCTATATCGCCCAAGAA**TGA**ATGCATTAG

Figure 4E: Sequence of Ad5/fib40-L chimeric fiber

ATGTTGTTGCAGATGAAGCGCGCAAGACCGTCTGAAGATACCTTCAACCCCGTGTATCCATATGAAC
ACTACAATCCCCTTGACATTCCATTTATTACACCCCCGTTTGCTTCCTCCAACGGCTTGCAAGAAAA
ACCTCCGGGAGTCCTCAGCCTGAAATACACTGATCCACTTACAACCAAAAACGGGGCTTTAACCTTA
AAATTGGGCACGGGACTAAACATTGATAAAAATGGAGATCTTTCTTCAGATGCTAGCGTGGAAGTTA
GCGCCCCTATCACTAAAACCAACAAAATCGTAGGTTTAAATTACACTAAGCCTCTCGCTCTGCAAAA
TAACGCGCTTACTCTTTCTTACAACGCGCCCTTTAACGTAGTAAATAATAATTTAGCTCTAAATATG
TCACAGCCTGTTACTATTAATGCAAACAACGAACTTTCTCTCTTAATAGACGCCCCACTTAATGCTG
ACACGGGCACTCTTCGCCTTCGAAGTGATGCACCTCTTGGACTAGTAGACAAAACACTAAAGGTTTT
GTTTTCTAGCCCCCTCTATCTAGATAATAACTTTCTTACACTAGCCATTGAACGCCCGCTAGCTCTA
TCCAGTAACAGAGCAGTGGCCCTTAAGTATTCACCACCTTTAAAAATAGAAAACGAAAACTTAACCC
TAAGCACAGGCGGACCTTTTACTGTAAGCGGGGGAAATTTAAACCTGGCAACATCGGCACCCCTCTC
CGTGCAAAACAATTCTCTCTCCTTAGGGGTTAACCCGCCTTTTCTCATCACTGACTCTGGATTAGCT
ATGGACTTAGGAGACGGTCTTGCATTAGGTGGCTCTAAGTTAATAATCAATCTTGGTCCAGGTTTAC
AAATGTCTAATGGAGCTATTACTTTAGCACTAGATGCAGCGCTGCCTTTGCAATATAAAAACAACCA
ACTTCAACTCAGAATTGGCTCCGCGTCTGCTTTAATTATGAGCGGAGTAACACAAACATTAAACGTC
AATGCCAATACCAGCAAAGGTCTTGCTATTGAAAATAACTCACTAGTTGTTAAGCTAGGAAACGGTC
TTCGCTTTGATAGCTGGGGAAGCATAGCTGTCTCACCTACTACCACTACCCCTACCACCCTATGGAC
CACCGCGGACCCGTCTCCTAACGCCACTTTTTATGAATCACTAGACGCCAAAGTGTGGCTAGTTTTA
GTAAAATGCAACGGCATGGTTAACGGGACCATATCCATTAAAGCTCAAAAAGGCACTTTACTTAAAC
CCACAGCTAGCTTTATTTCCTTTGTCATGTATTTTTACAGCGACGGAACGTGGAGGAAAAACTATCC
CGTGTTTGACAACGAAGGGATACTAGCAAACAGTGCCACATGGGGTTATCGACAAGGACAGTCTGCC
AACACTAACGTTTCCAATGCTGTAGAATTTATGCCTAGCTCTAAAAGGTATCCCAATGAAAAAGGTT
CTGAAGTTCAGAACATGGCTCTTACCTACACTTTTTTGCAAGGTGACCCTAACATGGCCATATCTTT
TCAGAGCATTTATAATCATGCAATAGAAGGCTACTCATTAAAATTCNCCTGGCGCGTTCGAAATAAT
GAACGTTTTGACATCCCCTGTTGCTCATTTTCTTATGTAACAGAACAA**TAA**ATGCATTAG

figures

fig 5

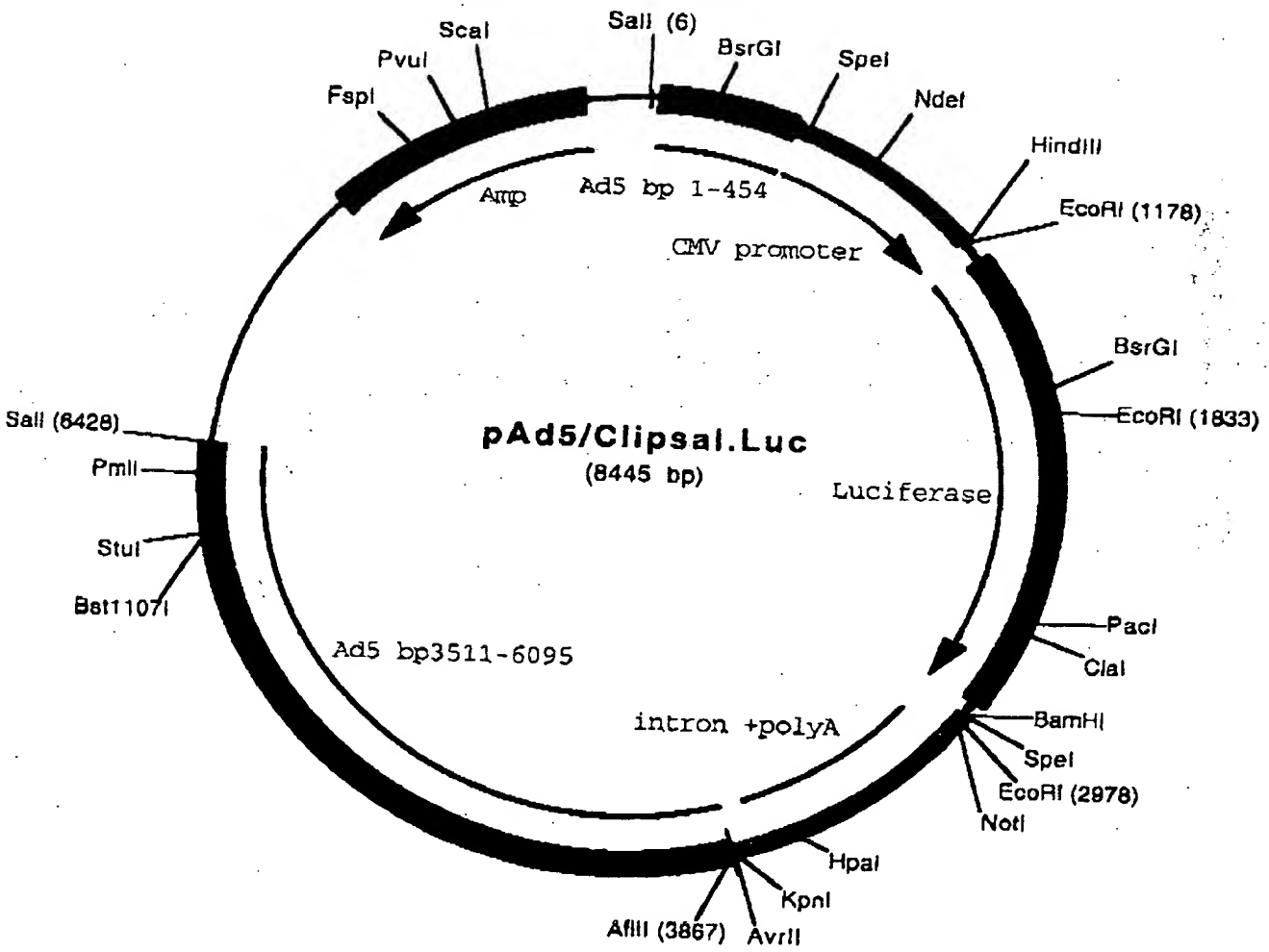
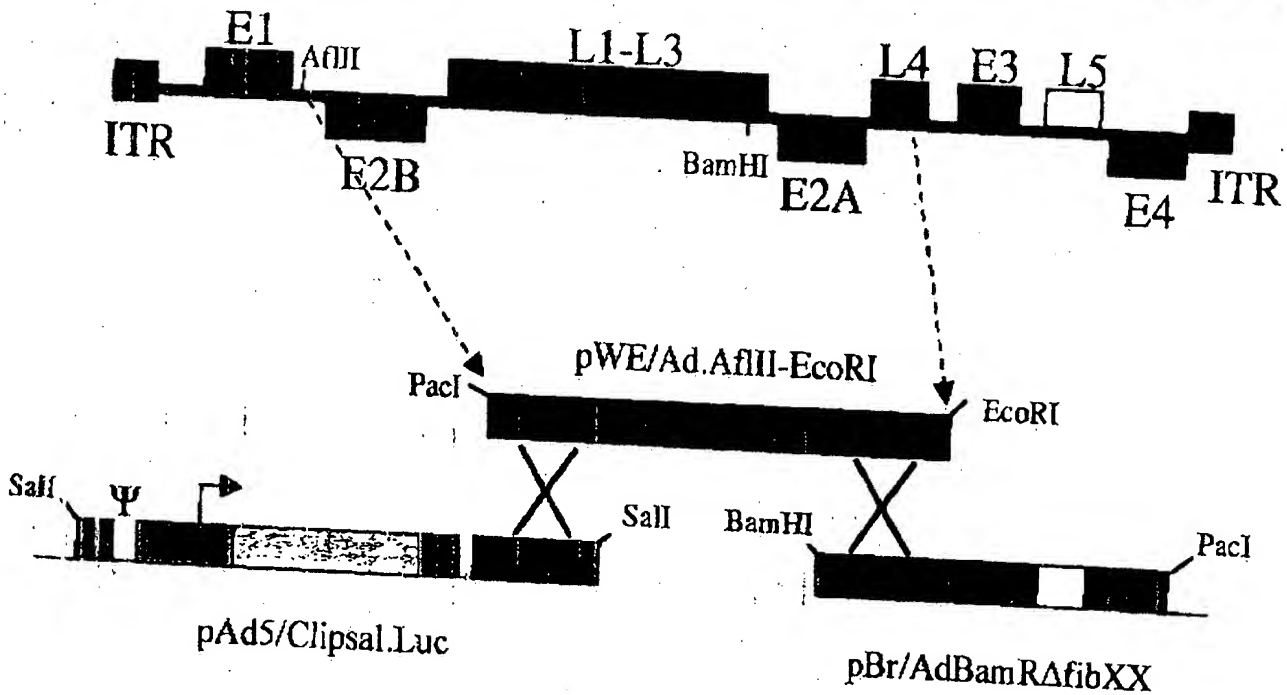


Figure 6: Generation of (chimaeric) adenoviruses



Cotransfection into PER.C6

Ad5.LucfibXX

Figure 7

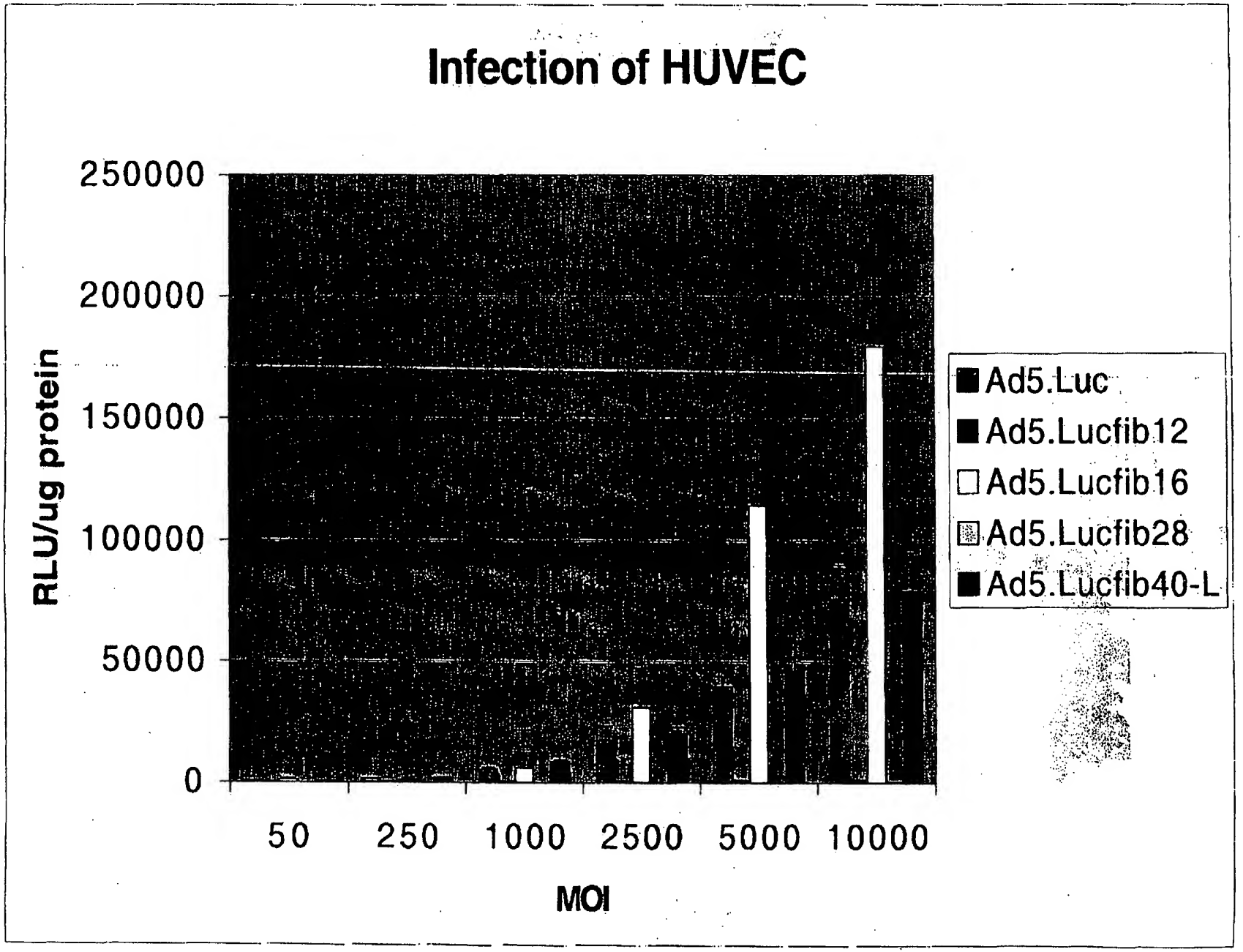


Figure 8

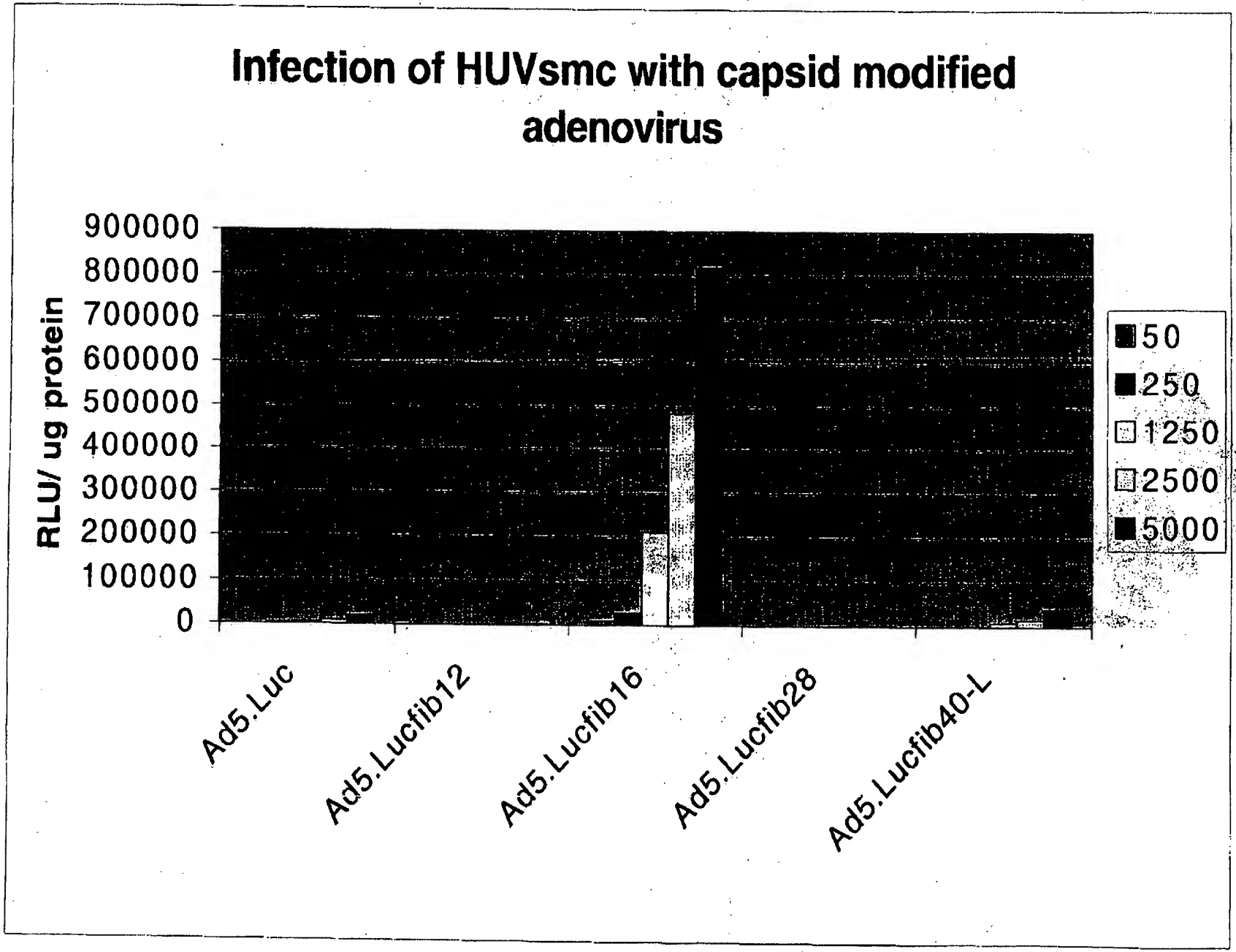


Fig 9A.

Alignment Report of Untitled, using Clustal method with Weighted residue weight table.
Thursday, November 19, 1998 18:25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1    ATGGC-----CAAACGAGCTCGGCTAAGCAGCT--------- Ad16 genbank.se
1    ATGTTGTTGCAGATGAAGCGCGCAAGACCGTCTGAAGATA Ad5/fib16.seq

29   CCTTCAATCCGGTCTACCCCTATGAAGATGAAAGCAGCTC Ad16 genbank.se
41   CCTTCAACCCCGTGTATCCATATGAAGATGAAAGCAGCTC Ad5/fib16.seq

69   ACAACACCCCTTTATAAACCCTGGTTTCATTTCCTCAAAT Ad16 genbank.se
81   ACAACACCCCTTTATAAACCCTGGTTTCATTTCCTCAAAT Ad5/fib16.seq

109  GGTTTTGCACAAAGCCCAGATGGAGTTCTAACTCTTAAAT Ad16 genbank.se
121  GGTTTTGCACAAAGCCCAGATGGAGTTCTAACTCTTAAAT Ad5/fib16.seq

149  GTGTTAATCCACTCACTACCGCCAGCGGACCCCTCCAACT Ad16 genbank.se
161  GTGTTAATCCACTCACTACCGCCAGCGGACCCCTCCAACT Ad5/fib16.seq

189  TAAAGTTGGAAGCAGTCTTACAGTAGATACTATCGATGGG Ad16 genbank.se
201  TAAAGTTGGAAGCAGTCTTACAGTAGATACTATCGATGGG Ad5/fib16.seq

229  TCTTTGGAGGAAAATATAACTGCCGCAGCGCCACTCACTA Ad16 genbank.se
241  TCTTTGGAGGAAAATATAACTGCCGAAGCGCCACTCACTA Ad5/fib16.seq

269  AAACTAACCACTCCATAGGTTTATTAATAGGATCTGGCTT Ad16 genbank.se
281  AAACTAACCACTCCATAGGTTTATTAATAGGATCTGGCTT Ad5/fib16.seq

309  GCAAACAAAGGATGATAAACTTTGTTTATCGCTGGGAGAT Ad16 genbank.se
321  GCAAACAAAGGATGATAAACTTTGTTTATCGCTGGGAGAT Ad5/fib16.seq

349  GGGTTGGTAACAAAGGATGATAAACTATGTTTATCGCTGG Ad16 genbank.se
361  GGGTTGGTAACAAAGGATGATAAACTATGTTTATCGCTGG Ad5/fib16.seq

389  GAGATGGGTTAATAACAAAAAAATGATGTACTATGTGCCAA Ad16 genbank.se
401  GAGATGGGTTAATAACAAAAAAATGATGTACTATGTGCCAA Ad5/fib16.seq

429  ACTAGGACATGGCCTTGTGTTTGACTCTTCCAATGCTATC Ad16 genbank.se
441  ACTAGGACATGGCCTTGTGTTTGACTCTTCCAATGCTATC Ad5/fib16.seq

469  ACCATAGAAAACAACACCTTGTGGACAGGCGCAAAACCAA Ad16 genbank.se
481  ACCATAGAAAACAACACCTTGTGGACAGGCGCAAAACCAA Ad5/fib16.seq

509  GCGCCAACTGTGTAATTAAAGAGGGAGAAGATTCCCCAGA Ad16 genbank.se
521  GCGCCAACTGTGTAATTAAAGAGGGAGAAGATTCCCCAGA Ad5/fib16.seq

549  CTGTAAGCTCACTTTAGTTCTAGTGAAGAATGGAGGACTG Ad16 genbank.se
561  CTGTAAGCTCACTTTAGTTCTAGTGAAGAATGGAGGACTG Ad5/fib16.seq

589  ATAAATGGATACATAACATTAATGGGAGCCTCAGAATATA Ad16 genbank.se
601  ATAAATGGATACATAACATTAATGGGAGCCTCAGAATATA Ad5/fib16.seq

629  CTAACACCTTGTTTAAAAACAATCAAGTTACAATCGATGT Ad16 genbank.seq
641  CTAACACCTTGTTTAAAAACAATCAAGTTACAATCGATGT Ad5/fib16.seq

669  AAACCTCGCATTTGATAATACTGGCCAAATTATTACTTAC Ad16 genbank.seq
681  AAACCTCGCATTTGATAATACTGGCCAAATTATTACTTAC Ad5/fib16.seq

709  CTATCATCCCTTAAAAGTAACCTGAACTTTAAAGACAACC Ad16 genbank.seq
721  CTATCATCCCTTAAAAGTAACCTGAACTTTAAAGACAACC Ad5/fib16.seq
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Fig 9A.

Alignment Report of Untitled, using Clustal method with Weighted residue weight table.
Thursday, November 19, 1998 18:26


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749   A A A A C A T G G C T A C T G G A A C C A T A A C C A G T G C C A A A G G C T T  Ad16 genbank.se
761   A A A A C A T G G C T A C T G G A A C C A T A A C C A G T G C C A A A G G C T T  Ad5/fib16.seq

789   C A T G C C C A G C A C C A C C G C C T A T C C A T T T A T A A C A T A C G C C  Ad16 genbank.se
801   C A T G C C C A G C A C C A C C G C C T A T C C A T T T A T A A C A T A C G C C  Ad5/fib16.seq

829   A C T G A G A C C C T A A A T G A A G A T T A C A T T T A T G G A G A G T G T T  Ad16 genbank.se
841   A C T G A G A C C C T A A A T G A A G A T T A C A T T T A T G G A G A G T G T T  Ad5/fib16.seq

869   A C T A C A A A T C T A C C A A T G G A A C T C T C T T T C C A C T A A A A G T  Ad16 genbank.se
881   A C T A C A A A T C T A C C A A T G G A A C T C T C T T T C C A C T A A A A G T  Ad5/fib16.seq

909   T A C T G T C A C A C T A A A C A G A C G T A T G T T A G C T T C T G G A A T G  Ad16 genbank.se
921   T A C T G T C A C A C T A A A C A G A C G T A T G T T A G C T T C T G G A A T G  Ad5/fib16.seq

949   G C C T A T G C T A T G A A T T T T T C A T G G T C T C T A A A T G C A G A G G  Ad16 genbank.se
961   G C C T A T G C T A T G A A T T T T T C A T G G T C T C T A A A T G C A G A G G  Ad5/fib16.seq

989   A A G C C C C C G G A A A C T A C C G A A G T C A C T C T C A T T A C C T C C C C  Ad16 genbank.se
1001  A A G C C C C C G G A A A C T A C C G A A G T C A C T C T C A T T A C C T C C C C  Ad5/fib16.seq

1029  C T T C T T T T T T C T T A T A T C A G A G A A G A T G A C T G A                 Ad16 genbank.se
1041  C T T C T T T T T T C T T A T A T C A G A G A A G A T G A C T G A                 Ad5/fib16.seq
```

Decoration 'Decoration #1': Box residues that differ from Ad16 genbank.seq.

Fig 9B

Alignment Report of Untitled, using Clustal method with PAM250 residue weight table.
Thursday, November 19, 1998 18:09

Page

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1   M A K R A R L S S - S F N P V Y P Y E D E S S S Q H P F I N Ad16 fiber protein GenBank
1   M - K R A R P S E D T F N P V Y P Y E D E S S S Q H P F I N Ad16A fib protein

30  P G F I S S N G F A Q S P D G V L T L K C V N P L T T A S G Ad16 fiber protein GenBank
30  P G F I S S N G F A Q S P D G V L T L K C V N P L T T A S G Ad16A fib protein

60  P L Q L K V G S S L T V D T I D G S L E E N I T A A A P L T Ad16 fiber protein GenBank
60  P L Q L K V G S S L T V D T I D G S L E E N I T A E A P L T Ad16A fib protein

90  K T N H S I G L L I G S G L Q T K D D K L C L S L G D G L V Ad16 fiber protein GenBank
90  K T N H S I G L L I G S G L Q T K D D K L C L S L G D G L V Ad16A fib protein

120 T K D D K L C L S L G D G L I T K N D V L C A K L G H G L V Ad16 fiber protein GenBank
120 T K D D K L C L S L G D G L I T K N D V L C A K L G H G L V Ad16A fib protein

150 F D S S N A I T I E N N T L W T G A K P S A N C V I K E G E Ad16 fiber protein GenBank
150 F D S S N A I T I E N N T L W T G A K P S A N C V I K E G E Ad16A fib protein

180 D S P D C K L T L V L V K N G G L I N G Y I T L M G A S E Y Ad16 fiber protein GenBank
180 D S P D C K L T L V L V K N G G L I N G Y I T L M G A S E Y Ad16A fib protein

210 T N T L F K N N Q V T I D V N L A F D N T G Q I I T Y L S S Ad16 fiber protein GenBank
210 T N T L F K N N Q V T I D V N L A F D N T G Q I I T Y L S S Ad16A fib protein

240 L K S N L N F K D N Q N M A T G T I T S A K G F M P S T T A Ad16 fiber protein GenBank
240 L K S N L N F K D N Q N M A T G T I T S A K G F M P S T T A Ad16A fib protein

270 Y P F I T Y A T E T L N E D Y I Y G E C Y Y K S T N G T L F Ad16 fiber protein GenBank
270 Y P F I T Y A T E T L N E D Y I Y G E C Y Y K S T N G T L F Ad16A fib protein

300 P L K V T V T L N R R M L A S G M A Y A M N F S W S L N A E Ad16 fiber protein GenBank
300 P L K V T V T L N R R M L A S G M A Y A M N F S W S L N A E Ad16A fib protein

330 E A P E T T E V T L I T S P F F F S Y I R E D D . Ad16 fiber protein GenBank
330 E A P E T T E V T L I T S P F F F S Y I R E D D . Ad16A fib protein
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Decoration 'Decoration #1': Box residues that differ from the Consensus.